VOLUME 87

ACTA PHYSIOLOGICA SCANDINAVICA

Vol 87 INDEX

Fasc 1 (January 1973)

Abstracts from Meeting of the Scandinavian Physiological Society in Uppsala 24—25 November 1972

Demonstrations

Communications

Fasc 2 (February 1973)

Fasc 3 (March 1973)

Short Communications

Fasc 4 (April 1963)

Supplements

INDEX AUCTORUM

KUNGL. BOKTR. STOCK OLM 1 73

Acta physiol scand 1973 87 1—14

From the Brain Research Laboratory E Blocket the University Hospital of Lund
and from the Department of Surgery the University of Lund Sweden

The Effect of Hypercapnic Acidosis upon the Energy Metabolism of the Brain in Arterial Hypotension Caused by Bleeding

By

BO EKLOF VERNON MACMILLAN[1] and BO K SIESJO

Received 29 March 1972

Abstract

EKLOF B V MACMILLAN and B K SIESJO *The effect of hypercapnic acidosis upon the energy metabolism of the brain in arterial hypotension caused by bleeding* Acta physiol scand 1973 *87* 1—14

The effect of hypercapnia upon the sensitivity of the cerebral energy metabolism to low perfusion pressures was studied by decreasing the mean arterial blood pressure to 60 and 45 mm Hg respectively in rats maintained at arterial CO_2 tension of 60—65 mm Hg The hypercapnia gave rise to a moderate extra and intracellular acidosis The cerebral venous P_{O_2} rose and remained above 50 mm Hg even in the hypotensive groups In spite of this venous hyperoxia a reduction of the mean arterial blood pressure to 60 mm Hg gave rise to slight but consistant changes in the tissue concentrations of adenine nucleotides At a pressure of 45 mm Hg there was a major derangement of the energy state of the tissue in 4 out of 6 animals It is concluded that hypercapnic acidosis adversely affects the cerebral energy metabolism in hypotensive states probably by inducing a flow pattern which leads to regional ischemia

In a preceding communication experiments were described in which the cerebral energy state and the extra and intracellular pH were determined in rats after a reduction of the mean arterial blood pressure to 60 and 45 mm Hg respectively (Eklof MacMillan and Siesjo 1972) In some of these hypotensive animals there were moderate changes in the lactate content and in the concentrations of adenine nucleotides but in general the experiments corroborated the well established autoregulatory ability of the cerebral circulation (Lassen 1959 Rapela and Green 1964 Häggendal and Johansson 1965 Harper 1966 Häggendal *et al* 1970) In most of the studies of the cerebral circulation (CBF) during states of decreased cerebral perfusion pressure the CBF has remained close to normal until perfusion pressures of 50—60 mm Hg have been reached but since major biochemical signs of a disturbed

[1] R S McLaughlin Fellow on leave of absence from the Department of Medicine (Neurology) Toronto General Hospital Toronto Canada.

energy metabolism do not appear until the perfusion pressure falls to 35—40 mm Hg (Siesjo and Zwetnow 1970 a and b) it may be deduced that the tissue can tolerate a certain decrease in CBF below the normal

We have observed (unpublished experiments) that in many animals an essentially normal cerebral energy state is obtained even if the blood pressure is reduced to about 30 mm Hg for 30 min while in others the energy metabolism fails at much higher pressures This suggests that other mechanisms than the perfusion pressure and the depth of anesthesia (*cf* Nilsson and Siesjo 1970 Nilsson 1971) determine the outcome of the hypotension A previous series of preliminary experiments in dicated that hypercapnia may adversely affect the energy state of the tissue in hypotensive conditions (Siesjo *et al* 1971) suggesting that tissue acidosis may be one of the factors accounting for the variability of results

The present experiments had the objective of studying the effects of moderate *hypercapnia upon the energy metabolism of the brain in arterial hypotension To* that end the blood pressure was reduced to 60 and 45 mm Hg respectively (see Eklof MacMillan and Siesjo 1972) in animals maintained at a Pa_{CO} of 60—65 mm Hg and measurements were made of arterial and cerebral venous P_O P_{CO_2} and pH, of extra and intracellular acid base parameters and of tissue parameters allowing an assessment of the cerebral energy state

Methods

he experimental procedures analytical methods and calculations used here were identical to *ose described in the preceding communication (Eklof MacMillan and Siesjo 197?)* In ummary male Wistar rats (300—400 g) which were immobilized and lightly anesthetized with ›6 Halothane were made hypercapnic and then bled to mean arterial blood pressures of 60 and 45 mm Hg respectively The animals were first allowed a steady state period at normal Pa_{CO_2} levels (35—40 mm Hg) Hyperventilation was then started and enough carbon dioxide was delivered to the inlet gate of the respirator to maintain Pa_{CO_2} at 60—65 mm Hg Five to ten min later the bleeding was begun When the desired blood pressure was obtained which required 5—10 min the blood pressure was maintained constant for another 30 min Thus the total hypercapnic period was 45—50 min while the period of stable hypotension was 30 min A normotensive control group was obtained by increasing the arterial CO_2 tension to 60—65 mm Hg without arterial bleeding In this group the blood pressure was allowed to attain its spontaneous level (see also below) The body temperature was maintained close to 37 C and enough oxygen was administered to maintain Pa_O well above 100 mm Hg

At each pressure level two parallel series of animals were run In the A groups blood was sampled from the femoral artery and from the superior sagittal sinus for measurements of P_O P_{CO_2} and pH and cisternal CSF was sampled for measurements of P_{CO} In the B series the superior sagittal sinus was not exposed CSF was sampled for measurements of total CO lactate pyruvate and glucose and the tissue was frozen *in situ* for subsequent measurements of phosphocreatine ATP ADP AMI glucose G 6 P pyruvate lactate α ketoglutarate malate and glutamate Phosphocreatine and the adenine nucleotides were measured in order to assess the energy state of the tissue lactate and pyruvate to evaluate any activation of glycolysis and to allow derivation of cytoplasmatic NADH/NAD ratios and the other metabolites to gain information on metabolic flu c and on the pattern of a possible substrate depletion

No normotensive control group was run for metabolite analyses since such a group wa made simultaneously in the laborat n in the ourse of another study (6 % CO_2 see Siesjo Fol bergrová and MacMillan 197? I lbergrová MacMillan and Siesjo 1972 a and b) However this group was analysed somewh t later then the hypotensive groups described in this and in the preceding communication d a slight change in the analytical procedure for ADP during that time interval had what altered the ADP values (by about 10 o) and the calculated energy charge potential by about 0 3) In order to avoid erroneous conclusions

TABLE I Mean arterial blood pressure arterial P_{CO_2} P_{O_2} and pH body temperature and blood hemoglobin concentration in the two series of normotensive and hypotensive rats In the A series blood was sampled from the femoral artery and from the superior sagittal sinus for measurements of P_{CO_2} P_{O_2} and pH In the B series the sinus was not exposed and the tissue was frozen for measurements of metabolites Means ± S E.

Group	MABP mm Hg	P_{CO_2} mm Hg	P_{O_2} mm Hg	pH	Temp C	Hb g/100 ml
A n=6	115 ±2	63.7 ±1.6	138 ±4	7.24 ±0.01	37.0 ±0.1	15.8 ±0.2
B n=7	124 ±4	60.7 ±0.4	125 ±4	7.26 ±0.01	36.8 ±0.1	16.0 ±0.3
A n=7	60	64.8 ±0.6	160 ±2	7.12 ±0.02	37.0 ±0.2	14.6 ±0.7
B n=6	60	65.2 ±0.5	162 ±4	7.14 ±0.02	37.3 ±0.1	14.2 ±0.5
A n=6	45	64.4 ±0.4	155 ±6	7.01 ±0.01	37.2 ±0.1	13.1 ±0.6
B n=6	45	65.3 ±0.9	161 ±8	7.04 ±0.01	37.2 ±0.2	13.1 ±0.2

the present "hypercapnic" values for ATP ADP and AMP were therefore compared to the corresponding values obtained both in the hypercapnic control group (Folbergrová MacMillan and Siesjo 1972 a) and in the previous normocapnic control group (Eklof MacMillan and Siesjo 1972) Since hypercapnia does not alter the concentrations of the tissue adenine nucleotides (Folbergrova MacMillan and Siesjo 1972 a) this comparison should remove the possibility of obtaining falsely significant changes in the hypotensive hypercapnic animals

Statistical analysis Wilcoxon rank test was performed on the experimental groups compared to the control group The following symbols for statistical significance are used $p < 0.05$* $p < 0.01$ ** $p < 0.001$***

Results

Table I shows that the A and B groups were similar with respect to arterial blood pressure arterial P_{CO_2} P_{O_2} and pH body temperature and arterial hemoglobin *concentration*

Group A

Table II illustrates the arteriovenous differences for P_{CO_2} P_{O_2} and pH measured at the end of the experiments Hypercapnia alone decreased the normal arteriovenous P_{CO_2} difference of 10—11 mm Hg (Eklof MacMillan and Siesjo 1972) to about 7 mm Hg and increased the venous P_{O_2} from a normal value of about 40 mm Hg to about 70 mm Hg An induced decrease in the mean arterial blood pressure to 60 and 45 *mm* Hg *gave rise to increased arteriovenous* P_{CO_2} differences (10.5 and 15.4 mm Hg respectively) but the widening of the arteriovenous P_{CO_2} differences was less marked than during normocapnia (see Eklöf MacMillan and Siesjo 1972)

TABLE II P_{CO_2} P_{O_2} and pH in the femoral artery and the superior sagittal sinus as well as the corresponding a v differences measured at 30 min in the control group and in the groups bled to 60 and 45 mm Hg respectively Means ± S E

MABP mm Hg	P_{CO_2} mm Hg			P_{O_2} mm Hg			pH		
	art	vein	diff	art	vein	diff	art	vein	diff
115 ±2 n=6	63 7 ±1 6	70 6 ±2 3	6 9 ±1 1	138 ±4	69 9 ±3 5	67 9 ±5 0	7 244 ±0 014	7 219 ±0 012	0 024 ±0 004
60 n=7	64 8 ±0 6	75 3 ±0 7	10 5** ±0 3	160 ±2	61 6 ±2 1	98 9*** ±2 7	7 118 ±0 017	7 093 ±0 014	0 026 ±0 006
45 n=6	64 4 ±0 4	79 7** ±0 8	15 4*** ±0 8	155 ±6	54 8*** ±1 9	100 0*** ±4 2	7 013*** ±0 007	6 986*** ±0 006	0 027 ±0 002

In Fig 1 the arterial and cerebral venous P_{CO} values measured before the induction of the hypercapnia have been compared to the corresponding values measured 10, 20 and 30 min respectively after the desired blood pressure levels had been obtained Also shown in the figure are the mean tissue CO_2 tensions calculated from the arterial and the cerebral venous P_{CO_2}s (see below) Since the arterial CO tension was maintained constant and since the arteriovenous P_{CO} differences increased in the hypotensive groups there was a slight progressive increase in the calculated tissue P_{CO_2} in the 60 and 45 mm Hg hypotensive groups The venous P_{O_2} values fell the hypotensive groups but it should be remarked that the venous P_O measured the hypercapnic group maintained at 45 mm Hg was clearly higher than the

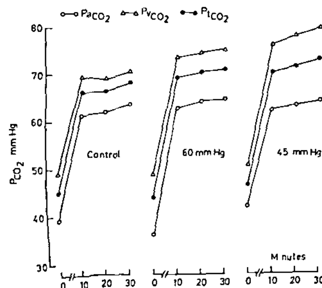


Fig 1 Arterial and venous Pco measured in the femoral artery and in the superior sagittal sinus and the calculated tissue CO_2 tension before induction of hypercapnia (time 0) and at 10 20 and 30 min respectively after the desired blood pressure level was obtained

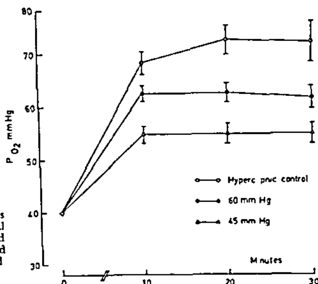


Fig 2 Venous oxygen tensions measured in the superior sagittal sinus at times 0 and 10 20 and 30 min after the desired blood pressure level had been obtained Means ± S E

venous P_{O} measured in a normocapnic control group with a normal blood pressure (about 40 mm Hg see preceding paper) Fig 2 shows that the venous P_{O_2} values measured at times 10 20 and 30 min respectively did not vary at any of the blood pressure levels The lowest venous P_{O} measured in any of the hypotensive hypercapnic was 47 mm Hg illustrating the venous hyperoxia elicited by the increased CO tension

In order to assess the tissue (and CSF) CO tensions the mean tissue CO tension was calculated by adding 1 mm Hg to the arithmetic mean of the arterial and the cerebral venous P_{CO} (Ponten and Siesjo 1966 *cf* Eklöf MacMillan and Siesjo 1972) and the CO tensions thus calculated were compared to those measured in cisternal CSF (Table III) There were no significant differences between the two CO tensions in any of the experimental groups The mean differences between the calculated tissue CO tensions and the measured arterial CO_2 tensions were therefore used to calcuate tissue CO_2 tensions in the individual experiments of group B

TABLE III Comparison between calculated tissue CO tension (Pt_{CO}) and CO tension measured in CSF in control group and in hypotensive groups Means ± S E

P_{CO} mm Hg	Control	60 mm Hg	45 mm Hg
Calculated tissue P_{CO}	68 2 ±1 9	70 2 ±0 3	73 0 ±0 5
Measured CSF P_{CO}	65 1 ±1 6	69 4 ±0 7	72 8 ±0 9

TABLE IV Measured CO_2 content in CSF with calculated CSF values for P_{CO_2} $[HCO_3^-]$ and pH in the control group and in the two hypotensive groups Means ± S E

CSF				
MABP mm Hg	P_{tCO_2} mm Hg	TCO_2 meq/kg	$[HCO_3^-]$ meq/kg	pH
124 ±4 n=7	65 7 ±0 4	32 3 ±0 4	30 3 ±0 4	7 291 ±0 001
60 n=6	71 5*** ±0 5	33 0 ±0 8	30 8 ±0 3	7 262*** ±0 006
45 n=6	74 0*** ±0 [illegible]	32 0 ±0 5	29 6 ±0 5	7 231*** ±0 009

Group B

Table IV shows the calculated CO_2 tensions the measured CO_2 contents and the derived HCO_3^- concentrations and pH values in cisternal CSF Hypercapnia alone decreased the CSF pH from the normocapnic value of about 7 10 (Siesjo Folbergrova and MacMillan 1972) to 7 29 In the hypotensive groups there was a further fall in the CSF pH but since the HCO_3^- concentration remained unchanged this fall was due to the increase in the CO_2 tension The figures of Table IV thus show that there was a moderate to marked extracellular acidosis in all hypercapnic groups

When the tissue concentrations of phosphocreatine ATP ADP AMP lactate and pyruvate were measured it became apparent that hypercapnia drastically af

TABLE V Brain tissue concentrations (mmol/kg wet tissue) of phosphocreatine ATP ADP AMP For the control group and the 60 mm Hg group the means standard errors and ranges group

Group	PCr	ATP	ADP	AMP
Control	4 65	3 0[illegible]	0 26	0 039
	±0 04	±0 0[illegible]	±0 01	±0 001
n=6	4 [illegible]1 – 4 76	2 94 – 3 08	0 24 0 27	0 035 – 0 043
60 mm Hg	4 44	2 95	0 34***	0 044
	±0 1[illegible]	±0 04	±0 01	±0 003
n=6	3 [illegible]5 – 4 67	2 81 – 3 07	0 32 – 0 37	0 034 – 0 0[illegible]
45 mm Hg	1 [illegible]	2 44	0 72	0 38
	1 01	1 2[illegible]	1 30	1 18
	0 90	0 99	1 13	1 01
	4 72	2 83	0 32	0 051
	4 90	2 90	0 39	0 0[illegible]4
	0 [illegible]7	0 57	0 [illegible]8	1 37
Mean	2 29	1 83 **	0 81***	0 67[illegible]***
S E	±0 81	±1 02	±0 16	±0 239

fected the energy state of the group maintained at a mean arterial blood pressure of 45 mm Hg However the variability was so marked that the results may be more meaningfully described in terms of the pattern seen in the individual experiments Table V therefore lists the means standard errors and ranges for the control group and for the 60 mm Hg group and in addition the individual results from the brains of the 45 mm Hg group Apart from the concentrations of the above metabolites the table also gives the calculated energy charge potentials (ECP) and the lactate/pyruvate ratios In the 60 mm Hg group all ECP values were lower than the lowest value in the control group The same conclusion can be drawn if the values for the 60 mm Hg groups are compared to the corresponding values obtained in the normocapnic control group of the preceding communication (Eklof MacMillan and Siesjo 1972 ECP range 0 941—0 949) In the 45 mm Hg group 4 out of 6 animals showed grossly abberant values for PCr ATP ADP and AMP and in these brains there were marked increases in lactate and in the lactate/pyruvate ratio as well In the remaining two animals of this group the ECP values were lower than the lowest value in the control group and the lactate contents and lactate/pyruvate ratios were higher than the highest value of that group In summary then it appeared that in the hypercapnic group maintained at 60 mm Hg the energy state of the brain as judged from the calculated ECP value was moderately but significantly altered in comparison to the control group In the 45 mm Hg group two animals fell in the above category while the remaining four showed a grossly deranged energy state

In order to allow calculation of cytoplasmatic NADH/NAD ratios and comparison of substrate levels in the experimental groups the intracellular lactate pyruvate and glucose concentrations were calculated on the assumption of a 3 % blood and 15 % extracellular volume (*cf* Eklof MacMillan and Siesjo 1972) Table VI shows that the decrease in the mean arterial blood pressure gave rise to marked increases

lactate and pyruvate as well as calculated lactate/pyruvate ratios and energy charge potentials ar l ted wh le the ind v dual values means and standard errors are gi en for the 45 mm Hg

ECP	La	Py	La/Py
0 950 ±0 001 0 947–0 952	0 90 ±0 04 0 79–1 05	0 063 ±0 003 0 054–0 074	14 3 ±0 2 13 6–14 8
0 394** ±0 003 0 930–0 938	1 43 ±0 41 0 87–3 45	0 069 ±0 006 0 058–0 096	19 4 ±3 4 14 1–35 9
0 791	14 95	0 141	106
0 505	23 98	0 048	500
0 495	22 62	0 070	323
0 934	1 46	0 065	22 5
0 925	1 66	0 063	26 3
0 363	25 27	0 043	589
0 669*** ±0 100	14 99 ±4 49	0 072 ±0 014	211 1 * ±78 1

TABLE VI Lactate and pyruvate concentrations measured in blood and CSF and calculated intracellular concentrations in the control group and in the 2 hypotensive groups Means ± S E

MABP mm Hg	Lactate mEqv/kg			Pyruvate mEqv/kg		
	Blood	CSF	I c	Blood	CSF	I c
124 ±4 n=6	1 21 ±0 23	1 91 ±0 08	0 94 ±0 08	0 080 ±0 006	0 124 ±0 006	0 068 ±0 005
60 n=6	4 91*** ±0 56	2 59*** ±0 14	1 46 ±0 61	0 152 ±0 008	0 132 ±0 005	0 073 ±0 009
45 n=6	8 48*** ±0 20	3 62*** ±0 26	23 26*** ±7 31	0 175 ±0 011	0 135 ±0 006	0 076 ±0 074

in the blood lactate and pyruvate concentrations to moderate increases in the CSF lactate concentrations but to no significant increases in the CSF or intracellular pyruvate concentrations There was a clear disparity between the increase in the calculated intracellular lactate concentration and the increase in the CSF lactate concentration Thus, in those four animals in which there was a marked increase in the calculated intracellular lactate concentration (*23 36 38 and 40 mmol/kg of i c water*) the CSF lactate content was increased by only 0 5—1 5 mmol/kg in comparison to the remaining two animals The results suggest that the increase in the tissue lactate content occurred in parts of the brain some distance away from

TABLE VII Measured concentrations of glucose (mmol/kg) in blood CSF and brain tissue as well as calculated intracellular glucose concentration The table also gives the glucose concentration ratios i c fluid to CSF i c fluid to blood and CSF to blood Means and standard errors are listed for the control group and the 60 mm Hg group while individual values means and standard errors are given for the 45 mm Hg group

MABP mm Hg	Blood	CSF	Tissue	I c	I c /CSF	I c /Blood	CSF/Blood
124 ±4 n=6	9 62 ±0 83	6 27 ±0 48	4 34 ±0 36	5 10 ±0 45	0 81 ±0 04	0 53 ±0 02	0 66 ±0 02
60 n=6	21 20*** ±1 09	7 50 ±0 29	7 01*** ±0 34	8 60*** ±0 50	1 15 ±0 07	0 41 ±0 02	0 36 ±0 02
45	25 57	9 36	5 77	5 90	0 63	0 23	0 37
	24 66	8 75	2 32	0 44	0 05	0 02	0 35
	26 51	8 18	2 19	0 27	0 03	0 01	0 31
	24 71	9 10	8 79	10 96	1 20	0 44	0 37
	28 29	8 51	9 00	11 27	1 32	0 40	0 30
	29 36	9 32	1 85	0			0 31
Mean S E M	26 52 ±0 79	8 87** ±0 19	4 99 ±1 37	4 81 ±2 19	0 65 ±0 27	0.22 ±0 09	0 34 ±0 01

TABLE VIII Measured T_{CO} content in the brain tissue (mEqv/kg) and calculated intracellular parameters [HCO_3^-] pH (derived from the CO_2 method see text) lactate/pyruvate ratio and cytoplasmatic NADH/NAD$^+$ ratio In the control group and the 60 mm Hg group are means and standard errors listed while in addition the individual results are given in the 45 mm Hg group

Group	Tco	[HCO⁻]	pH(co)	La/Py	NADH NAD
Control	16 4	15 0	6 987	13 8	1 5
n=6	±0 3	±0 4	±0 009	±0 7	±0 1
60 mm Hg	17 7	16 9	6 996	17 3	2 0
n=6	±0 4	±0 6	±0 016	±4 4	±0 6
45 mm Hg	16 5	15 1	6 94	173	17
	10 4	5 2	6 45	1076	32
	7 8	1 5	5 97	537	5 0
	17 8	17 8	7 00	18 6	2 1
	18 7	18 5	7 01	23 3	2 7
	9 6	4 2	6 40	1421	39
Mean	13 5	10 4	6 67	525 *	16***
S E	±1 9	±3 1	±0 18	±240	±7

cisternal CSF The implication of this is that neither will the measured CSF lactate concentrations reflect extracellular changes in those ischemic parts of the brain nor will the calculation of the intracellular lactate concentrations indicate the true increase in intracellular lactate in the ischemic and nonischemic parts of the brain (see Discussion)

Table VII illustrates the changes in the blood CSF and intracellular glucose concentrations A decrease in mean arterial blood pressure to 60 mm Hg gave rise to a doubling of the glucose concentration of blood but to relatively moderate increases in the CSF glucose and in the calculated intracellular glucose The individual values of the 45 mm Hg group showed that there was very little if any intracellular glucose calculated for three of the animals These were the brains in which an excessive lactate accumulation occurred and in which the ECP values were very low Since there are reasons to believe that the derangement of the tissue energy state is grossly inhomogenous in such brains the very low intracellular glucose concentration may indicate the cell glucose concentration only in part of the tissue (see below)

The inhomogenous derangement of the energy metabolism of the brain suggested by the above results and by other considerations (see Discussion) hampers the calculation of intracellular pH values and cytoplasmatic NADH/NAD ratios The values given below will therefore be quantitatively meaningful only for the 60 mm Hg group while those given for the 45 mm Hg group will be useful merely as rough approximations to the true events However since the total CO content and the tissue metabolites were measured on separate hemispheres the results also provide some information on the equality of changes between the two hemispheres Tab VIII shows that there were no significant changes in the calculated pH values in the lactate/pyruvate and NADH/NAD ratios between the control group an

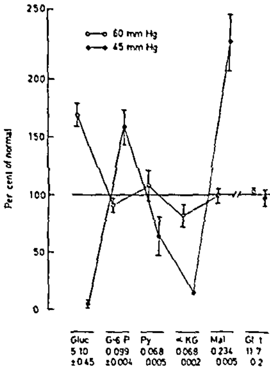


Fig 3 Comparison of intracellular concentrations of glucose G-6 P pyruvate α ketoglutarate malate and glutamate between control group and groups lled to mean arterial blood pressures of 60 and 45 mm Hg respectively Mean values in per cent of the listed control values ± S E. In the 45 mm Hg group only those three rats which had signs of pronounced brain ischemia were included

oup maintained at 60 mm Hg in blood pressure In the latter group only two animals had intracellular lactate concentrations lactate/pyruvate ratio and NADH/NAD ratios which were higher than the highest values in the control group Since all six of these animals had lowered ECP value the results corroborate those obtained in normocapnia (see Eklof MacMillan and Siesjo 1972) in indicating that a change in the energy charge potential precedes detectable increases in the lactate concentration or in the NADH/NAD ratio

In the group maintained at a mean arterial blood pressure of 45 mm Hg two of the animals had normal (calculated) pH values and only slightly elevated lactate/pyruvate and NADH/NAD ratios and those were the animals with only slight increases in the lactate content and moderate decreases in ECP (see Table V) In three of the remaining animals there were very marked decreases in the measured CO content and in the calculated [HCO₃] and pH In these animals which showed excessive change in lactate and ECP (Table V) the calculated lactate/pyruvate and NADH NAD ratios were also markedly elevated In general then when the measurement on one hemisphere showed a pronounced derangement of the energy state the other hemisphere showed a marked fall in total CO content Thus although the values of Table VIII do not quantitatively reflect the true tissue changes they indicate the gross degree of derangement in the acid base and redox states and they suggest that the changes observed affected both hemispheres about equally

The pronounced variability in results in the 45 mm Hg group and the proposed inhomogeneity in distribution of the biochemical lesions also complicate the description of changes occurring in substrate levels Fig 3 shows the changes (in percentage) in the cellular concentrations of glucose G 6-P pyruvate α ketoglutarate malate and glutamate in the hypotensive groups compared to the normotensive control group However in order to illustrate the changes occurring in pronounced ischemia only those three animals which showed the largest changes were included in the 45 mm Hg hypotensive group In the group maintained at 60 mm Hg in blood pressure the only conspicuous change was an increase in the glucose concentration (*cf* Eklof MacMillan and Siesjö 1972) to about 170 % of the control value However in the three ischemic brains belonging to the group of animals bled to a blood pressure of 45 mm Hg there were marked changes in substrate levels Thu glucose fell to less than 10 % of normal pyruvate and especially α ketoglutarate fell and there were pronounced increases in the malate concentration

Discussion

The present results have demonstrated that when the mean arterial blood pressure is decreased to low values during hypercapnia a more pronounced derangement of the cerebral energy metabolism occurs than during normocapnia and that the cerebral energy metabolism fails in spite in very high cerebral venous oxygen tensions There are two possible explanations for the results (1) that CO as such or the accompanying changes in the extra or intracellular pH interfere with cerebral energy metabolism in such a way that higher than normal tissue oxygen tensions are required and (2) that the hypercapnic acidosis changes the flow pattern in the tissue in such a way that regional ischemia occurs The first of those possibilities may be excluded on grounds that during normotensive conditions up to 40 % CO may be administered to rats without causing a derangement of the cerebral energy state (Siesjo Folbergrova and MacMillan 1972 Folbergrova MacMillan and Siesjö 1972 a and b) Furthermore if the arterial P_O is lowered to below 30 mm Hg hypercapnia of the same degree as used here is without effect on the energy state of the tissue (MacMillan and Siesjo 1972 a and b see also Gottesfeld and Miller 1969) We are therefore left with the tentative conclusion that the hypercapnic acidosis gives rise to regional ischemia when the perfusion pressure is lowered (Siesjö Eklof and MacMillan 1972)

It has previously been assumed that cerebral function and cerebral energy metabolism fail when the cerebral venous P_{O_2} falls to certain critical levels whether this fall is caused by hypoxemia or by a reduction in the cerebral perfusion pressure (Opitz and Schneider 1950 Thews 1960 1963) It is implicit in this assumption that the cerebral blood flow declines in a homogenous fa hion throughout the tissue and that no islands of total ischemia develop The complete dissociation between venous P_O and cerebral energy state observed in the present hypercapnic material does in fact suggest that a pattern of patchy perfusion is created Thus

as long as some parts of the tissue have a high perfusion the venous P_O measured remains high but the presence of unperfused areas gives signs of a deranged cerebral energy state According to this hypothesis (see Siesjo, Eklof and MacMillan 1972) the cerebral energy pattern observed in the tissue represents a mixture of normal areas and areas which take on the metabolic pattern of total ischemia

Although the dissociation between the venous P_{O_2} and the energy state by itself indicates the presence of patchy perfusion and regional total ischemia we may use the metabolic results to support the hypothesis It is well known that in ischemia a depletion of ATP is preceded by a fall in the PCr concentration (Lowry *et al* 1964 Schmahl *et al* 1965, 1966) It has further been shown that when pH_i' is reduced during hypercapnic acidosis there is a fall in the PCr concentration in accordance with a pH dependent shift in the creatine phosphokinase (CPK) equilibrium (Siesjo, Folbergrova and MacMillan 1972 see also Rose 1968) We may use the ATP, ADP and pH_i values for the ischemic animals in the group maintained at a blood pressure of 45 mm Hg and calculate using the CPK equilibrium the PCr concentrations which would be expected in a homogenous system In all instances the PCr concentrations measured were much too high to fit a CPK equilibrium The values become more meaningful if we assume that the final PCr ATP and ADP concentrations represent a mixture of areas some of which may be totally ischemic and others which may be adequately perfused It should be added that if the system is inhomogenous in the sense that widely different concentrations of PCr ATP and ADP occur in various parts of the tissue the CPK method cannot be used to calculate pH' In fact it may indicate an alkaline shift in pH when there is gross acidosis

Although the present results give good evidence of an inhomogenous fall in CBF when the perfusion pressure is decreased during hypercapnia the results give no clue to whether the inhomogeneity involves gross tissue regions or if it occurs as a disseminated change in the microcirculation Previous morphological studies have shown that ischemia often preferentially affects the border zones between the territories of the major cerebral arteries (Lindenberg 1963 Romanul and Abramowicz 1964 Brierley *et al* 1969) and it is conceivable that total ischemia may occur in such areas under the present experimental conditions In this context it should be mentioned that hypercapnia has been reported to shunt blood away from diseased areas in the brain (intracranial steal see Brawley Strandness and Kelly 1967 Hoedt Rasmussen *et al* 1967 Symon 1968 Fieschi *et al* 1968) Whatever is the pattern of distribution of the ischemic lesions the present results seem to establish that hypercapnia adversely affects the flow pattern within the tissue at low perfusion pressures It is tempting to assume that this effect is due to extracellular acidosis and vasodilatation (Severinghaus and Lassen 1968 Lassen 1968) disturbing the adaptation of the local circulation to a critically low perfusion pressure

This study was supported by grants from the Swedish Medical Research Council (Project No B72 14X 2179 04 and B72 14X 263 08B) from the Swedish Bank Tercentenary Fund by U S PHS Grant No RO1 NS 07838 03 from NIH and by Tore Nilsons Fund

References

BRAWLEY B W D F STRANDNESS JR and W A KELLY The physiologic response to experimental cerebral ischemia *Arch Neurol* 1967 *17* 180—187

BRIERLEY J B A W BROWN B J EXCELL and B S MELDRUM Brain damage in the rhesus monkey resulting from profound arterial hypotension I Its nature distribution and general physiological correlates *Brain Research* 1969 *13* 68—100

EKLOF B N MACMILLAN and B K SIESJO Cerebral energy state and cerebral venous Po in experimental hypotension caused by bleeding *Acta physiol scand* 1972 *86* 515—527

FIESCHI C A AGNOLI N BATTISTINI L BOZZAO and M PRENCIPE Derangement of regional cerebral blood flow and of its regulating mechanisms in acute cerebrovascular lesions *Neurology* 1968 *18* 1166—1179

FOLBERGROVA J V MACMILLAN and B K SIESJO The effect of moderate and marked hypercapnia upon the energy state and upon the cytoplasmatic NADH/NAD ratio of the rat brain *J Neurochem* 1972 a Submitted for publication

FOLBERGROVA J V MACMILLAN and B K SIESJO The effect of hypercapnic acidosis upon some glycolytic and Krebs cycle associated intermediates in the rat brain *J Neurochem* 1972 b Submitted for publication

GOTTESFELD Z and A T MILLER JR Metabolic response of rat brain to acute hypoxia influence of polycythemia and hypercapnia *Amer J Physiol* 1969 *216* 1374—1379

HAGGENDAL E and B JOHANSSON Effects of arterial carbon dioxide tension and oxygen saturation on cerebral blood flow autoregulation in dogs *Acta physiol scand* 1965 *66* Suppl 258 27—53

HAGGENDAL E J LOFGREN N J NILSSON and N N ZWETNOW Effects of varied cerebrospinal fluid pressure on cerebral blood flow in dogs *Acta physiol scand* 1970 *79* 262—271

HARPER A M Autoregulation of cerebral blood flow influence of the arterial blood pressure on the blood flow through the cerebral cortex *J Neurol Neurosurg Psychiat* 1966 *29* 398—403

HOEDT RASMUSSEN K E SKINHOJ O PAULSEN J EWALD and N A LASSEN Regional cerebral blood flow in acute apoplexy *Arch Neurol* 1967 *17* 271—281

LASSEN N A Cerebral blood flow and oxygen consumption in man *Physiol Rev* 1959 *39* 183—238

LASSEN N A Brain extracellular pH The main factor controlling cerebral blood flow *Scand J clin Lab Invest* 1968 *22* 247—251

LINDENBERG R Patterns of CNS vulnerability in acute hypoxemia including anaesthesia accidents In *Selective vulnerability of the brain in hypoxemia* Ed J P Schade and W H McMenemey Blackwell Scientific Publ Oxford 1963 189—209

LOWRY O H J V PASSONNEAU F X HASSELBERGER and D W SCHULTZ Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain *J biol Chem* 1964 *239* 18—30

MACMILLAN V and B K SIESJO Brain energy metabolism in hypoxemia *Scand J clin Lab Invest* 1972 a Submitted for publication

MACMILLAN V and B K SIESJO Intracellular pH of the brain in arterial hypoxemia evaluated with the CO_2 method from the creatine phosphokinase equilibrium *Scand J clin Lab Invest* 1972 b Submitted for publication

NILSSON L The influence of barbiturate anaesthesia upon the energy state and upon acid base parameters of the brain in arterial hypotension and in asphyxia *Acta neurol scand* 1971 *47* 233—253

NILSSON L and B K SIESJO The effect of anesthetics upon labile phosphates and upon extra and intracellular lactate pyruvate and bicarbonate concentrations in the rat brain *Acta physiol scand* 1970 *80* 235—248

OPITZ E and M SCHNEIDER Über die Sauerstoffversorgung des Gehirns und den Mechanismus von Mangelwirkungen *Ergeb Physiol* 1950 *46* 126—260

PONTEN U and B K SIESJO Gradients of CO_2 tension in the brain *Acta physiol scand* 1966 *67* 129—140

RAPELA C E and H D GREEN Autoregulation of canine cerebral blood flow *Circulat Res* 1964 *15* Suppl 1 205—211

ROMANUL F and H ABRAMOWICZ Changes in brain and pial vessels in arterial border zones *Arch Neurol* 1964 *11* 40—65

ROSE J A The state of magnesium in cells as estimated from the adenylate kinase equilibrium *Proc nat Acad Sci* 1968 *60* 235—248

SCHMAHL F W E BETZ H TALKE and H J HOHORST Energiereiche Phosphate und

Metabolite des Energiestoffwechsels in der Grosshirnrinde der Katze *Biochem Z* 1965 *342* 518—531

SCHMAHL F W E BETZ E DETTINGER and H J HOHORST Energiestoffwechsel der Grosshirnrinde und Elektroencephalogramm bei Sauerstoffmangel *Pflugers Arch ges Physiol* 1966 *292* 46—59

SEVERINGHAUS J W and N A LASSEN Step hypocapnia to separate arterial from tissue P_{CO_2} in the regulation of cerebral blood flow *Circulat Res* 1967 *20* 272—278

SIESJO B K and N N ZWETNOW The effect of hypovolemic hypotension on extra and intra cellular acid base parameters and energy metabolites in the rat brain *Acta physiol scand* 1970 a *79* 114—124

SIESJO B K and N N ZWETNOW Effects of increased cerebrospinal fluid pressure upon adenine nucleotides and upon lactate and pyruvate in rat brain tissue *Acta neurol scand* 1970 b *46* 187—202

SIESJO B K L NILSSON M ROKEACH and N N ZWETNOW Energy metabolism of the brain at reduced cer bral perfusion pressures and in arterial hypoxemia. In *Brain Hypoxia* Ed J B Brierley & B S Meldrum S I M P /William Heinemann Medical Books Ltd London 1971

SIESJÖ B K B EKLOF and V MACMILLAN Energy metabolism in the brain in ischemia Princeton Conference on Cerebrovascular disease Princeton 1972 In press

SIESJÖ B K J FOLBERGROVA and V MACMILLAN The effect of hypercapnia upon intra cellular pH in the brain evaluated with the CO_2 method and from the creatine phospho kinase equilibrium *J Neurochem* 1972 Submitted for publication

SYMON L Experimental evidence for "intracranial steal following CO_2 inhalation *Scand J clin Lab Invest* 1968 Suppl 102 XIII A

THEWS G Die Sauerstoffdiffusion im Gehirn *Pflugers Arch ges Physiol* 1960 271 197—226

THEWS G Implications to physiology and pathology of oxygen diffusion at the capillary level In *Selective Vulnerability of the Brain* Ed J P Schade and W H McMenemey Blackwell Scientific Publ Oxford 1963 pp 27—35

Acta physiol scand 1973 87 15—26

From the Department of Physiology Karolinska Institutet Stockholm Sweden

Nonlinear Dynamic Properties of a Somatomotor Reflex System A Model Study

By

Erik Borg

Received 5 april 1972

Abstract

Borg E *Nonlinear dynamic properties of a somatomotor reflex system A model study* Acta physiol scand 1973 *87* 15—26

The time course of polysynaptic reflex responses is known to exhibit two prominent non linearities one dependent on the amplitud of the stimulus and the other dependent on the direction of change of the stimulus Both nonlinearities were demonstrated in the open loop responses of the acoustic middle ear reflex and simulated in a piecewise linear model Their significance for stability of the closed loop feed back system was investigated In this model the source of nonlinearity was as a pool of "reflex units with various thresholds and time constants The model responses were studied in the time domain and the frequency domain The *amplitude dependent nonlinearity could be simulated when low threshold reflex units were* made slow and tonic and high threshold units fast and tonic or when some of the high threshold units were phasic A decrease in transport delay at increased input step amplitude was seen under these conditions but also when all units mere made equal Both types of nonlinearities were found to improve the stability of the model system without decreasing its bandwidth (a measure of the speed of action) The nonlinearities were suggested to be important in systems with high values of transport delay

In a linear system the response to the sum of two stimuli is identical to the sum of the responses of the two stimuli presented individually Most biological systems can be regarded as linear only in certain conditions *e g* for small variations in amplitude Deviations from linearity can have many courses and causes Threshold and saturation phenomena are important features of biological systems Both give rise to non linearity In addition to these generally occurring nonlinearities there are others which have been recognized only lately (Clynes 1961 1962 Borg 1972 c) Two types of nonlinear behaviour will be investigated in the present study a) that dependent on stimulus amplitude and b) that dependent on the direction of change of stimulus amplitude None of them are necessarily followed by nonlinear steady state stimulus response functions and both may only appear in the time course of the response The amplitude dependent nonlinearity shows up as a longer rise time for responses to weak stimuli than to strong ones This amplitude dependence seems to be common in polysynaptic somatomotor reflex systems (see *e g* Sherrington 1911 Wersall 1958) and has recently been analysed in some detail in the acoustic

Metabolite des Energiestoffwechsels in der Grosshirnrinde der Katze *Biochem Z* 1965 *342* 518—531

SCHMAHL F W E BETZ E DETTINGER and H J HOHORST Energiestoffwechsel der Grosshirnrinde und Elektroencephalogramm bei Sauerstoffmangel *Pflugers Arch ges Physiol* 1966 *292* 46—59

SEVERINGHAUS J W and N A LASSEN Step hypocapnia to separate arterial from tissue P_{CO_2} in the regulation of cerebral blood flow *Circulat Res* 1967 *20* 272—278

SIESJO B K and N N ZWETNOW The effect of hypovolemic hypotension on extra and intra cellular acid base parameters and energy metabolites in the rat brain *Acta physiol scand* 1970 a *79* 114—124

SIESJÖ B K and N N ZWETNOW Effects of increased cerebrospinal fluid pressure upon adenine nucleotides and upon lactate and pyruvate in rat brain tissue *Acta neurol scand* 1970 b *46* 187—202

SIESJO B K L NILSSON M ROKEACH and N N ZWETNOW Energy metabolism of the brain at reduced cerebral perfusion pressures and in arterial hypoxemia. In *Brain Hypoxia* Ed J B Brierley & B S Meldrum S I M P /William Heinemann Medical Books Ltd London 1971

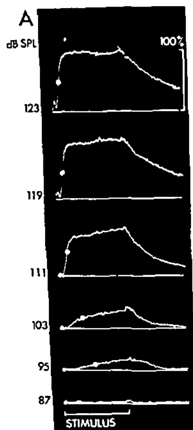
SIESJO B K B EKLOF and V MACMILLAN Energy metabolism in the brain in ischemia Princeton Conference on Cerebrovascular disease" Princeton 1972 In press

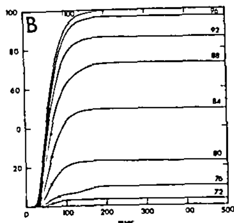
SIESJO B K J FOLBERGROVA and V MACMILLAN The effect of hypercapnia upon intra cellular pH in the brain evaluated with the CO_2 method and from the creatine phospho kinase equilibrium *J Neurochem* 1972 Submitted for publication

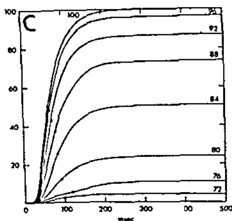
SYMON L Experimental evidence for intracranial steal following CO inhalation *Scand J clin Lab Invest* 1968 Suppl 102 XIII A

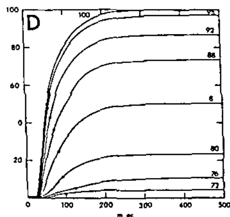
THEWS G Die Sauerstoffdiffusion im Gehirn *Pflugers Arch ges Physiol* 1960 *271* 197—226

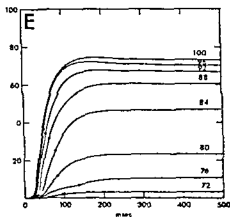
THEWS G Implications to physiology and pathology of oxygen diffusion at the capillary level In *Selective Vulnerability of the Brain* Ed J P Schade and W H McMenemey Blackwell Scientific Publ Oxford 1963 pp 27—35











0 04 s and 0 01 s for high threshold units E Low threshold units are tonic (0 03 s) and high threshold units are phasic (0 015 s and 0 03 s) In B C D and E the thresholds follow a Gaussian frequency function of intensity and the black dots indica e ris tim t> ,0

The response of the middle ear reflex in the nonanesthetized rabbit
The technique for stimulating and recording the middle ear reflex activity in the present study was the same as that described earlier (Møller 1961 Borg 1972 a 1972 b) In the present study a series of responses to 0 5 s bursts of 0 5 kHz pure tones of various intensities were observed under open loop conditions The stimulus tone was presented to one ear wherein both middle ear muscles had been inactivated earlier The responses were obtained from the contra lateral ear wherein both muscles were intact

Results

Open loop system

The aim of the first series of experiments was to determine the composition of the reflex unit pool that approximated the amplitude dependent nonlinearity as it appears in the acoustic middle ear reflex open loop responses

Fig 2 shows a comparison between open loop responses of the middle ear reflex (A) and those of the model with various combinations of reflex unit properties (B C D E) In all cases the stimuli were steps with different amplitudes The open loop responses of the crossed middle ear reflex were obtained as changes in the acoustic impedance of the ear *in a nonanesthetized rabbit (Fig 2 A) The stimuli were* contralateral tone bursts at 0 5 kHz In B all reflex units of the model were tonic and had identical time constants 0 02 s This model was thus approximately linear In C all units were tonic with the time constants decreasing from 0 04 s to 0 01 s with increasing thresholds In D there were two types of tonic units with time constants of 0 04 s (low threshold) and 0 01 s (high threshold) In E the low threshold units were tonic and every second reflex unit with a threshold above 84 arbitrary stimulus units was phasic all tonic reflex units had time constants equal to 0 03 s wereas all phasic reflex units had constants of 0 015 s and 0 03 s In all of the models of Fig 2 the thresholds of the reflex units followed the Gaussian frequency function The dots white in A and black in B C D and E indicate the time that was necessary to reach a response of 50 per cent of maximal amplitude for each step response (rise time)

It can be seen in Fig 2 A that the rise time (white dots) of the responses of the physiologic system decreased when the stimulus amplitude was increased There was a concomittant decrease of the latency The response of the models (Fig 2 B C D E) showed a decrease in both latency and rise time in all cases A small *decrease in latency and rise time was seen also in the linear model* (B all reflex units equal) The decrease of latency is probably a consequence of the exponential *build up of the level of excitation in the reflex unit pool (output of* leaking integrator Fig 1) The small decrease in rise time in the linear model (B) is probably a consequence of this decrease in latency It can be noted in Fig 2 B C and E (stimulus amplitude 76) that one unit was triggered after a long delay giving

Fig 2 Open loop step responses of biologic system (A) and model systems (B C D E) A Impedance change in one ear having both middle ear muscles intact to stimulation of the other ear with a 0 5 s burst of 0 5 kHz pure tone Both middle ear muscles were cut in stimulated ear *White dots show rise time to 50 % of steady state amplitude* B All reflex units have equal time constants (0 02 s) Input step amplitudes are shown by legend numbers Relative response amplitude on ordinate C The time constants decrease continuously from 0 04 s for low threshold units to 0 01 s for high threshold units D Time constant for low threshold units 1

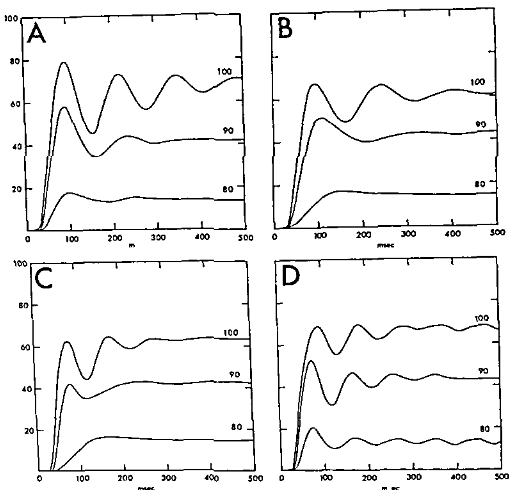


Fig 3 Closed loop step responses of model system (gain constant = 1 0) A B C Closed loop responses of systems the open loop step responses of which are shown in Fig 2 B C and D D Low threshold tonic units are fast (0 02 s) and high threshold tonic units are slow (0 04 s) illustrating the parallel action of m stapedius (low threshold fast) and of m tensor tympani (high threshold slow) S shaped stimulus-response curve

stability was improved at high amplitude as compared to a model with all reflex units equal (Fig 3 A)

Direction sensitive properties Different dynamic properties for positive and negative step inputs or changes in input amplitude is a characteristic nonlinear property of the middle ear reflex system (see Fig 2 A) In the case of the middle ear reflex a positive step corresponds to the onset of a tone burst and a negative step may correspond to the decay of a tone burst The influence of unidirectional rate sensitive properties on the stability of the model is illustrated in Fig 4 All reflex units were tonic and had equal time constants for increasing amplitude in Fig 4 A

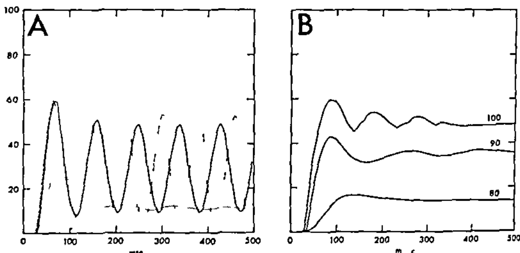


Fig 4 Direction dependent properties of closed loop system A Tonic units with time constants for decrease in amplitude equal to time constants for increasee in amplitude (———) one fourth ( ) four times the time constants for increase ( ) B Step responses of closed loop system to three stimulus amplitudes Tonic units (low threshold) and phasic units (high thresholds) *cf* open loop responses in Fig 2 E

The time constants for decreasing amplitude were (a) equal to those for the onset (continuous line), (b) one fourth (broken line) or (c) four times (dotted line) the onset time constants There was a notable improvement of the stability when the dynamic properties were made slower for decreasing than for increasing amplitude The 50 per cent rise time was unchanged

Fig 4 B shows closed loop responses of the model system composed of tonic and phasic units the open loop responses of which are illustrated in Fig 2 E The decay was equal for the phasic and the tonic units Also in this case the oscillations decreased signifying an improvement of stability (*cf* Fig 3 A)

The frequency domain transfer functions showed the increase in stability as a decrease of the amplitude of the resonance peaks At high input amplitude the band width (a measure of the speed of the system) was the same or even slightly increased in the nonlinear systems as compared to the variant with all units equal (Fig 3 A) At low input amplitude however the nonlinear systems were slower (except for Fig 3 D) At an input amplitude of 100 the bandwidth was 9 7 Hz 9 2 Hz and 10 5 Hz and the peak amplitude was 7 3 dB 4 3 dB and 1 9 dB for the cases shown in Fig 3 A B and Fig 4 B respectively

Transport delay A transport delay can be expressed in linear transfer functions as an increase in phase shift that is proportional to frequency An increase in phase shift is known to decrease the stability The important consequences of latency on the stability of the closed loop system of the model is shown in Fig 5 This figure shows step responses of the closed loop system with all time constants equal to 0 01 s (A) and 0 1 s (B) The delay in the feed back loop was 0 01 s 0 025 s and 0 1 s for

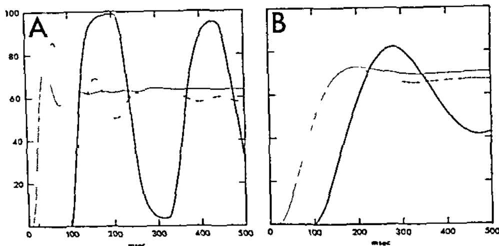


Fig 5 Influence of transport delay on stability of closed loop model system Transport delay 0 1 s (continuous line) 0 025 s (broken line) 0 01 s (dotted line) Number of activated units is proportional to input amplitude implying a linear stimulus response curve A Tonic units with time constants equal to 0 01 s B Tonic units with time constants equal 0 1 s

the dotted the broken and the continuous curve respectively It is seen in Fig 5 A and B that the amplitude of the oscillations increased and the frequency decreased when the transport time was increased A comparison of Fig 5 A and B shows that the relative amplitude of the oscillations decreased when the system was made slower which means that the stability was improved (B)

Discussion

The present model study based on a piecewise linear approximation of a nonlinear system illustrates certain functional characteristics that could not be elucidated in linear analysis and modeling of reflex systems The main finding was an improvement in the stability of the closed feed back system when nonlinear with respect to amplitude or direction of change of amplitude

Validity of the model The fundamental component of the model the reflex unit cannot be thought of as having any simple anatomical counterpart and was introduced for the purpose of piecewise linearization The concept is based on observations on the middle ear reflex system (Borg 1971) with regard to the time course of responses to small step stimuli superimposed on controlled background activity in the reflex arc Even though the steady state amplitude of the step responses were the same the onset became faster as the level of the background activity was increased This increase in speed was tentatively explained as in part due to a recruitment of faster motor units at higher stimulus intensities The reflex unit of the model probably represents the activity of interneurons as well as motoneurons Furthermore the all-or none character of the reflex units assumed in the

model implies a simplification of the properties of the motor units According to several studies the motoneurons of limb muscles respond with a nearly constant dis charge rate within a large portion of their dynamic range for voluntary activation Only at the weakest and the strongest powers the firing rates are graded (Bigland and Lippold 1954 Clamann 1970) It is well known that rate of electrical stimula tion of muscle nerves has great influence on the time course of the muscle contraction up to and even beyond fusion frequency (see *e g* Buller and Lewis 1965 Fuchs and Luscher 1971) The time course of the contraction is therefore likely to be changed at both ends of the dynamic range of the contraction In case firing rate can be regarded approximately constant in the major part of the dynamic range the all-or none property of the reflex unit will accurately describe the motor unit

The firing rate of motoneurons in the middle ear muscles has not been analysed sufficiently to serve as the basis of a decision on this point Although the literature reveals several values of impulse frequency the authors often have pointed out the questionableness of the values Based on EMG recordings of the m tensor tympani in the cat Okamoto *et al* (1954) differentiated between phasic and tonic motor units and stated maximal impulse frequencies of approximately 25 and 18 Hz respectively Also from EMG recordings in the cat Eliasson and Gisselsson (1955) observed only insignificant dependence on stimulus sound intensity and mentioned a maximal frequency of 25 to 30 Hz for both muscles In the EMG of the m stapedius of the rabbit Wersall (1958) could demonstrate single units firing from 10 to 42 Hz but this was regarded not to be the maximum Wigand (1965) showed recordings of one single unit in the nerve of the m stapedius of a rabbit having an impulse [illegible]ency from below 20 Hz up to about 100 Hz that was dependent on the frequen cy of the stimulus sound This range observed at a variation in sound frequency might be an expression for a considerable intensity dependence of the firing rate Thus gradation of impulse frequency is not experimentally settled On the other hand it is safer to state that variations in contraction times of single motor units occur and that this factor may be important with regard to the time course of the reflex response (Teig 1969 1972 Borg 1971)

Some observations point toward the existence of an upper limit for motor units in voluntary contractions above which firing ceases (Dasgupta and Simpson 1962) *This idea is lent support by a recent finding by Tan (1971) an increase of the* number of afferent neurons firing was shown to decrease and even stop the activity of low threshold tonic motor units

Inclusion of properties related to firing frequency and inhibition would have greatly increased the complexity of the model Since the model tested in the present study adequately described observed open loop reflex responses there is reason to believe that it also described the role of the nonlinearities for the closed loop system this despite the simplifications in the fundamental assumptions on the physiological origin of these nonlinearities

Functional significance of the nonlinearities The results of the present study show that both amplitude dependent and direction dependent nonlinearities improve the

stability of model systems with maintained bandwidth at high amplitudes of stimulation compared to a corresponding linear system (all reflex units equal) The cost of this increase in stability was that the speed of the system was decreased at low amplitude stimulation

The decrease of latency with more intense stimulation is due to the integration (Block A) before the threshold function (see also McRobert *et al* 1968) The concomitant decrease in phase shift stabilizes the system and thus allows the speed of the responses to increase (Fig 5) Improvement of the stability in certain unidirectional rate sensitive systems has also been discussed by Milsum (1969) and related to a decrease in phase shift

In biological systems the degree of stability if often decreased by the presence of transport delays (see Fig 5) Under such circumstances nonlinearities may counteract the adverse effect of the transport delay and be important in system stabilization

This study was supported by Karolinska Institutets Fonder Karolinska Institutet

References

BIGLAND B and O C J LIPPOLD Motor unit activity in the voluntary contraction of human muscle *J Physiol* (Lond) 1954 *125* 322—335

BORG E On the nonlinear dynamic properties of the acoustic middle ear reflex of unanaesthetized animals *Brain Res* 1971 *31* 211—215

BORG E On the change in the acoustic impedance of the ear as a measure of middle ear muscle reflex activity *Acta oto laryng* (Stockh) 1972 a *74* 163—171

BORG E On the use of the acoustic middle ear muscle reflexes in studies of auditory function in nonanesthetized rabbits *Acta oto laryng* (Stockh) 1972 b *74* In press

BORG E The dynamic properties of the acoustic middle ear reflex in nonanesthetized rabbits Quantitative aspects of a polysynaptic reflex system *Acta physiol scand* 1972 c *86* 366—387

BORG E On the neuronal organization of the acoustic middle ear reflex *Brain Res* 1972 d In press

BULLER A J and D M LEWIS The rate of tension development in isometric tetanic contractions of mammalian fast and slow skeletal muscle *J Physiol* (Lond) 1965 *176* 337—354

CLAMANN H P Activity of single motor units during isometric tension *Neurology* 1970 *20* 254—260

CLYNES M Unidirectional rate sensitivity A biocybernetic law of reflex and humoral systems as physiologic channels of control and communication *Ann N Y Acad Sc* 1961 *92* 946—969

CLYNES M The nonlinear biological dynamics of unidirectional rate sensitivity illustrated by analog computer analysis pupillary reflex to light and sound and heart rate behavior *Ann N Y Acad Sci* 1962 *98* 806—845

DASGUPTA A and J A SIMPSON Relation between firing frequency of motor units and muscle tension in the human *Electromyography* 1962 *2* 117—128

ELIASSON S and L GISSELSSON Electromyographic studies of the middle ear muscles of the cat *Electroceph clin Neurophysiol* 1955 *7* 399—406

FEDDE M R P D DEWET and R L KITCHELL Motor unit recruitment pattern and tonic activity in respiratory muscles of *Gallus domesticus* *J Neurophysiol* 1969 *32* 995—1004

FUCHS A F and E S LUSCHEI Development of isometric tension in simian extraocular muscle *J Physiol* (Lond) 1971 *219* 155—166

GRANIT R H D HENATSCH and G STEG Tonic and phasic ventral horn cells differentiated by post tetanic potentiation in cat extensors *Acta physiol scand* 1956 *37* 114—126

HENNEMAN E and C B OLSON Relations between structure and function in the design of skeletal muscles *J Neurophysiol* 1965 *28* 581—598

HENNEMAN E G SOMJEN and D O CARPENTER Excitability and inhibitibility of motoneurons of different sizes *J Neurophysiol* 1965 *28* 599—620

McROBERT H M E BRYAN and W TEMPEST The acoustic stimulation of the middle ear muscles *J Sound Vib* 1968 *7* 129—140

MILSUM J H The role of unidirectional rate sensitivity in biological control systems stabilization. *Ann N Y Acad Sci* 1969 *156* 731—739

MOLLER A R Bilateral contraction of the tympanic muscles in man examined by measuring acoustic impedance-change *Ann oto rhino laryng* 1961 *70* 735—755

OBERG P A and U SJOSTRAND Studies of blood pressure regulation III Dynamics of arterial blood pressure on carotid sinus nerve stimulation *Acta physiol scand* 1971 *81* 96—109

OKAMOTO M M SATO and I KIRIKAE Studies of the acoustic reflex Part II Experimental studies on the function of the tensor tympani muscle *Ann oto rhino laryng* 1954 *63* 950—959

SHERRINGTON C S *The integrative action of the nervous system* Constable & Co Ltd London 1911

TAN U Changes in firing rates of extensor motoneurones caused by electrically increased spinal inputs *Pflugers Arch ges Physiol* 1971 *326* 35—47

TEIG E Tension and contraction velocity of single motor units of the stapedius muscle *Acta physiol scand* 1969 *76* 16 A

TEIG E Tension and contraction time of motor units of the middle ear muscles in the cat. *Acta physiol scand* 1972 *84* 11—21

WERSALL R The tympanic muscles and their reflexes *Acta oto laryng* (Stockh) 1958 *Suppl 139* 1—112

WIGAND M E Der Stapediusreflex des wachen Kaninchens auf Tonreize mittlerer Lautstarke *Acta oto laryng* (Stockh) 1965 *59* 361—367

Acta physiol scand 1973 87 27—39

From the Department of Pediatrics Karolinska Institutet, St Goran s Children s Hospital and the Department of Physiology Gymnastik och Idrottshogskolan Stockholm Sweden

Effect of Physical Training on Hemodynamic Response during Submaximal and Maximal Exercise in 11-13-Year Old Boys

By

BENGT O ERIKSSON[1] and GUNTER KOCH[1]

Received 10 April 1972

Abstract

ERIKSSON B O and G KOCH *Effect of physical training on hemodynamic response during submaximal and maximal exercise in 11—13 year old boys* Acta physiol scand 1973 *87* 27—39

Nine healthy boys mean age 11 7 years (11 0—13 0) height 150 4 cm and weight 45 1 kg were examined with determinations of maximal oxygen uptake (1 85 l×min^{-1}) heart volume (499 ml) and total hemoglobin (391 g) Cardiac output was determined at rest and during exercise including maximal exercise using the dye dilution technique and i a. pressures were recorded Cardiac output was approximately 2 l×min^{-1} lower and the systemic a v oxygen difference (AVD) was correspondingly higher than for young adult men at the same submaximal oxygen uptake At maximal exercise cardiac output was 12 5 l×min^{-1} stroke volume 67 ml AVD 14 2 ml×100 ml^{-1} systolic diastolic and mean blood pressure 160 71 and 105 mm Hg respectively and total peripheral resistance 8 6 mm Hg×l^{-1}×min After a training period of 4 months a normal increase in height was found in all boys but body weight was unchanged Maximal oxygen uptake increased to 2 21 l×min^{-1} ($p < 0 01$) almost entirely due to increased stroke volume (80 ml) resulting in a maximal cardiac output of 14 6 l×min^{-1} No significant increase in AVD was found Mean blood pressure at maximal exercise increased significantly total peripheral resistance was unchanged Both heart volume and total hemoglobin showed minor increases but the changes found were not significantly larger than expected from body growth The hemoglobin concentration was normal (13 g %) for the age and unaffected by training

As demonstrated in Girl Swimmers (Åstrand *et al* 1963) physically well trained children can attain very high levels of maximal aerobic power Training studies of children have also demonstrated an increase in maximal aerobic power (Schwartz Britten and Thompson 1928 Ekblom 1969 a) The underlying mechanism for this increase is not known Judging from the change in heart volume which is supposed to be an indirect measure of stroke volume (Bevegard 1962) an increased strok

[1] Present addresses B O E Department of Pediatrics University of Gothenburg S 413 46 Gö teborg Sweden G K Department of Clinical Physiology Central Hospital K Sweden.

TABLE I Individual and mean values for some physiological and anthropometric data before (B) and after (A) physical training Stars indicates levels of significance for intra individual differances before and after training *** = p<0 001 ** = p<0 01 * = p<0 05

	Age Years		Height (H) m		Weight (W) kg		Heart volume (HV) ml	
	B	A	B	A	B	A	B	A
R F	11 2	11 8	1 411	1 440	47 8	46 7	375	365
J H	11 0	11 6	1 375	1 409	35 0	33 1	310	385
T L	11 9	12 5	1 614	1 668	50 7	53 8	625	690
A M	12 9	13 5	1 608	1 664	47 9	52 3	505	535
B M	11 5	12 1	1 614	1 655	57 3	55 0	600	705
G P	11 1	11 7	1 438	1 459	47 9	49 8	550	590
P P	13 0	13 6	1 527	1 568	43 1	47 5	630	770
Ro S	11 0	11 6	1 450	1 467	39 2	38 8	455	450
B Å	11 3	11 9	1 503	1 530	36 7	38 7	440	490
Mean	11 7	12 3***	1 504	1 540***	45 07	46 19	499	548**
S D	0 79	0 79	0 0923	0 1030	7 20	7 66	113 1	136 6

volume may be one of the mechanisms leading to increased maximal aerobic power

Very little is known about the adjustment of cardiac output and stroke volume at maximal exercise in children (Eriksson Grimby and Saltin 1971) Therefore it was felt that further information was needed regarding hemodynamic adjustment in children under different conditions including maximal exercise Since the subjects in the aforementioned study were in pubertal ages the present study was extended to lower age group Since it has been assumed that prepubertal training would yield the best result (Ekblom 1969 b) the effect of physical training on central circulation in children subjects were studied before and after physical training The purpose of this study was therefore to answer the following two questions

1 Do boys from 11—13 years differ from older boys and adults with respect to the adjustment of the central circulation during exercise?

2 How does physical training influence the adjustment of the central circulation during exercise?

The total investigation program also included an analysis of pulmonary gas exchange (Koch and Eriksson 1972 a) and pulmonary diffusing capacity (Koch and Eriksson 1972 b) the determination of respiratory and circulatory dimensions and of muscle mass (von Dobeln and Eriksson 1972) and determinations of muscle metabolism during exercise (Eriksson Gollnick and Saltin 1972)

Subjects

Nine boys 11—13 years of age volunteered with their parents consent for the study One other boy was examined before the training period but then had to be excluded because of a Salmonella infection All the boys were from the same elementary school some of them also belonged to a swimming club None of them undertook regular hard physical training in addition to the training program Individual values for age height and weight before and after training are given in Table I All the boys who completed the whole program had passed a medical examination prior to during and after the study and were found to be healthy

Total hemoglobin (Thb) g		Max O_2 — uptake $l \times min^{-1}$ STPD		Max heart rate beats × min^{-1}		Blood lactate concentration mM	
B	A	B	A	B	A	B	A
411	368	1 90	1 93	200	193	8 9	9 4
302	304	1 52	1 73	197	200	9 4	10 5
427	537	2 43	2 85	208	200	7 9	11 0
457	512	1 73	2 62	180	177	6 7	8 7
407	482	1 83	2 29	200	183	8 8	9 4
381	412	1 69	2 16	200	196	4 5	4 5
383	457	1 88	2 28	200	197	7 0	5 8
377	396	1 96	2 11	186	187	7 9	9 2
373	372	1 73	1 88	200	190	8 8	10 0
391	427	1 85	2 21**	196 8	191 4*	7 77	8 72*
43 2	75 9	0 25	0 36	8 5	7 9	1 52	2 16

Comments Representativity of the material

Since the number of subjects included in this study was rather small the representativity had to be established For this reason a control group of 16 boys from the same school and of the same age was examined Mean values for these boys were age 11 3 SD 0 3 years height 143 8 SD 5 7 cm weight 37 6 SD 5 5 kg maximal oxygen uptake 1 84 SD 0 27 $1 \times min^{-1}$ maximal HR 200 SD 6 beats × min^{-1} and maximal blood lactate concentration 8 6 SD 2 6 mM

The boys in the present study were 6 6 cm taller on the average than those in the control group Two boys were particularly tall for their age and contributed heavily *to the height discrepancy*

However normal height variation for this age group is rather large (Broman Dahlberg and Lichtenstein 1942) Thus mean height + 2 SD for 11 7 year old boys gives 160 cm and mean height—2 SD 132 cm Judging from the small difference in age *i e* 0 4 years between the studied group and the control group a height surplus of ? cm should be expected for the actual group

Values for maximal oxygen uptak were in good agreement *i e* 1 85 $1 \times min^{-1}$ and 1 84 $1 \times min^{-1}$ respectively this was also true of maximal heart rate (197 and 200 beats × min^{-1}) and maximal blood lactate (7 8 and 8 6 mM respectively) However maximal oxygen uptake per height squared (H^2) (von Dobeln and Eriksson 1972) separated the 2 groups the studied group having 0 82 and the control group 0 89 $1 \times min^{-1} \times m^{-2}$ This would imply that the studied group was somewhat less fit than normal contemporary boys Mean weight in the studied *group was 7 5 kg greater than in the control group* Even when considering body weight per height cubed the studied boys had a higher value *i e* 13 3 $kg \times m^{-3}$ and 12 6 $kg \times m^{-3}$ respectively This discrepancy was mainly due to 2 of the subjects whose weight exceeded the mean for Swedish boys in relation to height by +3 SD (*Broman Dahlberg and Lichstenstein* 1942) It thus appears that the studied group was not fully representative with respect to weight On the other hand physical training resulted in a weight normalization per height cubed after training this ratio had the same value as for the control group *i e* 12 6 *kg* × m^{-3}

Methods

Heart volume was determined in the prone position with biplane radiograms according to Kjellberg Rudhe and Sjostrand (1949) Total hemoglobin (Thb) was determined in duplicate by the alveolar CO method (Sjostrand 1948) Blood volume was calculated from Thb and the hemoglobin concentration in fingertip blood

Exercise *was performed on an electrically* braked bicycle ergometer (Elema) at a pedalling rate of 60 rpm Expired air was collected in Douglas bags and its volume measured in a Tissot spirometer A modified Haldane technique was used for gas analysis The variation coefficient

of the method were 1 4 % at submaximal loads and 0 8 % at maximal exercise Pairs of deter mination were 34 and 55 respectively Arterial blood lactate was determined with an en zymatic method (Scholz *et al* 1958) The dye dilution technique with indocyanine green (Cardiogreen) as the indicator and a Beckman densitometer as the recording unit were used to determine cardiac output This method has been widely used and comparative studies with other techniques of cardiac output determinations have demonstrated the reliability of the dye dilution technique (Hamilton *et al* 1948 Saltin 1972) Blood for three point calibration curves was obtained during maximal exercise in all subjects and the factors were around 1 1—1 2 The values for cardiac output were obtained after planimetrical determinations of the areas The error of the method for determination of cardiac output was calculated from the differences between 2 consecutive steady state determinations in which the mean difference *in heart rate did not exceed 4 beats × min ¹ The coefficient of variation was about 5 %* both at rest and during exercise Blood pressures were recorded with a pressure transducer (Elema 490 A) on a Mingograph 81 (Elema) Heart rate was determined from the ECG tracings Total peripheral resistance was calculated from cardiac output and mean blood pressure

Procedure

The boys were examined in November—December 1970 before and from the middle of May to the beginning of June 1971 after training Prior to the hemodynamic studies the boys maximal oxygen uptake was determined on 2 different days (precatheterization studies) The first day reactions to different loads were examined with the subjects exercising for 6 min at each load before maximal exercise was performed The second day 6 min of work at about 50 % of maximal intensity preceded maximal work

Heart rate was recorded each min during exercise and continuously at the end of maximal exercise Capillary blood for lactate determination was sampled from a fingertip at the end of and 3 min after maximal exercise Expired air was collected during the final min at the highest load double collections were made in all cases Maximal oxygen uptake was calculated as the mean of the values obtained the levelling-off criterion being used *i e* the point at which a further increase in the load fails to increase oxygen uptake

On the basis of these data a maximal load was chosen for each boy in the hemodynamic study which he could sustain for 5—7 min

Hemodynamic studies

The boys came to the laboratory in the morning Arterial and venous catheters were introduced aproximately 10 cm into cubital vessels using a modified Seldinger technique After a 10—20 min rest in a recumbent position expired air was collected for 10 min and 2 dye dilution curves plus blood pressure were simultaneously recorded With the subjects sitting on the bicycle a single determination of cardiac output was made blood pressures were recorded without ex pired air being collected in order to avoid orthostatic reactions All subjects worked at 250 *500 and 750 kpm × min ¹ Seven of them before training and eight after training also worked* at a higher load Maximal load was 856 kpm × min ¹ (range 750—1100) before and 939 (range 750—1200) kpm × min ¹ after training Work duration at submaximal loads was 7—9 min without interruption between the different loads However 15 min of rest was allowed between the highest submaximal and the highest maximal load Before starting maximal work the subjects exercised for 3 min at about 50 % of their maximal oxygen uptakes as a warming up procedure Work duration at maximal loads was 6—8 min 2 subjects before and 2 others after training only worked for 4—4 5 min

At submaximal work loads blood pressures were recorded in the 4th min of exercise while cardiac output and oxygen uptake were determined after 5 min In maximal exercise blood pressures were recorded during the third min Cardiac output and oxygen uptake determinations were made during the final min of exercise Each cardiac output determination included 2 dye-dilution curves except at rest in the sitting position Dye injections were performed at the same time as air collection At lower loads one sample of expired air was collected during the 2 dye injections providing a sampling time of about 2 min At higher submaximal load as well as at the maximal load one sample of expired air was taken per cardiac output deter mination The values stated for oxygen uptake in these cases are mean values This also applies to heart rate and cardiac output Arterial blood assay for lactate was taken at the end of exercise at 500 kpm × min ¹ and maximal exercise

Conventional statistical methods were used and Students t test was applied to the intra individual differences in comparing results before (= B) and after (= A) training

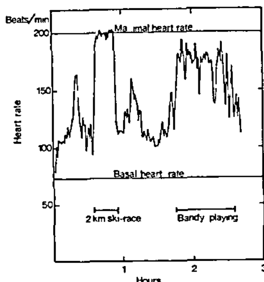


Fig. 1. Heart rate recordings of one subject (P.P.) during the training camp session using a tape recorder (SRA). The maximal heart rate obtained in the ergometer test is given in the figure as well as the basal heart rate.

Training program

Physical training was performed 3 times a week for 1 h per session under the supervision of a highly experienced physical education instructor. The training period lasted from the middle of January to the middle of May 1971. A total of 34 training sessions were held with a mean attendance of 29.8 sessions. Methods known to increase aerobic power were used. Thus most of the training consisted of high speed running from 3 up to 15 min repeated several times. When weather conditions were too poor training was held in a gymnasium in which the boys performed a vigorous gymnastic program. In April 1971 all the boys participated in a special training camp in the Swedish mountains where training was more frequent. Training there consisted mainly of cross country skiing twice a day. During this period heart rates were recorded using a tape recorder system (SRA) and blood lactate was determined after the training sessions in order to check training intensity. It was then found that heart rate (Fig. 1) and blood lactate often were above 170—180 beats$\times$min^{-1} and 5 mM respectively, indicating that the training demanded at least 85—90 % of the boys' maximal aerobic power.

Results

Individual and mean values as obtained during the precatheterization studies are given in Table I. During the study all the boys increased in height by an average of 3.6 cm ($p < 0.001$). However, mean weight only increased by 0.12 kg ($p > 0.05$). In fact 4 of the boys decreased in weight during the study despite a height increase of 1.7—4.1 cm. Maximal oxygen uptake increased from 1.85 l$\times$min^{-1} to 2.21 l$\times$min^{-1} ($p < 0.01$) and from 0.82 to 0.93 l$\times$min$^{-1}\times$m ($p < 0.01$). Maximal blood lactate concentration increased from 7.8 to 8.7 mM ($p < 0.05$), heart volume increased by 10 % to 548 ml ($p < 0.05$) and total hemoglobin increased by 9 % to 427 g ($p > 0.05$). Hemoglobin concentration decreased slightly (2 %) to 13.0 g %, resulting in a calculated 12 % rise in blood volume to 3.31 ($p < 0.05$).

Hemodynamic studies

Mean values for oxygen uptake, heart rate, cardiac output and blood pressure are

TABLE II Mean values and SD for oxygen uptake cardiac output heart rate blood pressures and blood lactate concentration at rest and during exercise before (B) and after (A) physical training Numbers of determinations = 9 except when indicated 1) n = 6 2) n = 8 3) n = 7 Stars indicates levels of significance for intra individual differances before and after training *** = $p<0\ 001$ ** = $p<0\ 01$ * = $p<0\ 05$

	Rest supine position		Rest sitting position	
	B	A	B	A
Oxygen uptake $l \times min^{-1}$ STPD	0 23 0 04	0 23 0 04		
Pulmonary ventilation $l \times min^{-1}$ BTPS	7 1 1 7	7 5 0 9		
Heart rate beats $\times min^{-1}$	81 6 8 1	71 2** 8 3	79 8 7 8	80 3 8 1
Cardiac output $l \times min$	5 0 0 9	5 1 1 0	3 9 0 6	4 5* 0 8
Stroke volume ml	62 2 12 4	72 4** 11 4	49 4 8 2	56 9* 13 1
Arterio venous oxygen difference ml $\times$ 100 ml $^{-1}$	4 6 0 7	4 5 0 4		
Systolic blood pressure mm Hg	119 7 8	123 8 1	115 10 1	126 9 5
Diastolic blood pressure mm Hg	65 4 9	65 7 1	66[1] 8 0	70 7 0
Mean blood pressure mm Hg	87 6 0	84 5 8	85 6 7	91 6 1
Total peripheral resistance mm Hg $\times (l \times min^{-1})^{-1}$	17 7 3 6	16 8 3 3	21 4 3 0	20 8 3 8
Blood lactate concentration mM	1 2 0 6	0 9 0 6		

given in Table II and mean or individual values for some of these variables are also displayed in Fig 2—5

Oxygen uptake at rest in a supine position was 0 23 $l \times min^{-1}$ During exercise at 250 and 500 kpm$\times min^{-1}$ it increased to 0 7 and 1 1 $l \times min^{-1}$ respectively thes values being practically identical before and after training Mean oxygen uptake a 750 kpm$\times min^{-1}$ before training was 1 54 $l \times min^{-1}$ However this load was not a true submaximal load for 2 of the subjects After training oxygen uptake at th equivalent load amounted to 1 73 $l \times min^{-1}$ The highest observed oxygen uptake it the hemodynamic study was 1 74 $l \times min^{-1}$ before and 2 10 $l \times min^{-1}$ after training ($p<0\ 001$)

Heart rate (Fig 2) at rest in the supine position decreased with training from 8

Exercise 250 kpm $\times$ min^{-1}		Exercise 500 kpm $\times$ min^{-1}		Exercise 750 kpm $\times$ min		Maximal exercise 856 kpm $\times$ min^{-1} (B) 939 kpm $\times$ min^{-1} (A)	
B	A	B	A	B	A	B	A
0 71 0 12	0 69 0 09	1 09 0 08	1 14 0 11	1 54 0 07	1 73* 0 09	1 74 0 22	2 10 ** 0 31
15 1 1 0	20 5* 4 9	25 6 3 6	32 1* 4 9	38 0 6 8	50 9** 8 6	47 8 8 1	69 6** 12 9
118 4 12 0	107 4 8 4	145 9 12 6	139 7* 12 2	172 0 8 9	168 0 13 6	186 6 8 2	184 6* 8 2
7 7 1 4	7 7 1 0	9 3 1 4	10 4 1 9	11 8 1 8	13 7* 1 6	12 5 2 5	14 6 * 3 2
65 3 10 0	72 8 12 5	64 1 11 4	75 7* 18 3	68 9 10 3	82 5 * 14 5	66 9 13 9	79 7* 19 9
9 3 1 2	9 0 1 3	12 7 2 3	11 1 1 4	13 3 2 0	12 7 1 6	14 2 2 1	14 7 1 6
132 13 1	140 17 3	145 10 9	153 17 2	158 11 8	160 21 5	160 12 5	173 19 1
68 8 2	68 9 0	67 6 2	72 11 7	68 4 5	77 8 5	71 5 3	84 6 0
88 9 6	96 9 1	94 8 6	102* 9 0	100 11 0	106 10 7	105 11 4	115 * 9 8
12 2 2 2	12 7 2 2	10 3 1 5	10 2 2 4	8 6 1 2	7 9 1 5	8 6 1 5	8 5 2 3
		1 8 0 9	1 8 0 9			5 6 1 9	6 3 2 0

to 71 beats $\times$ min^{-1} ($p < 0\ 01$) During submaximal exercise there was also a significant decrease averaging 7 beats $\times$ min^{-1} At maximal exercise there was virtually no change (187 and 185 beats $\times$ min^{-1}) before and after training respectively

Cardiac output (Fig 2 and 3) before *training was 5 0 l $\times$ min^{-1} at rest in the* supine position It dropped by 1 1 l $\times$ min^{-1} to 3 9 l $\times$ min^{-1} in the sitting position ($p < 0\ 001$) and increased with exercise to 7 7 9 3 and 11 8 l $\times$ min^{-1} at oxygen uptakes of 0 71 1 09 and 1 54 l $\times$ min^{-1} respectively Similar relationships between cardiac output and oxygen uptake were found with the subjects at rest and at the 2 lower submaximal work loads after training At 750 kpm $\times$ min^{-1} oxygen uptake was higher corresponding to a higher cardiac output (13 7 l $\times$ min^{-1}) In maximal exercise after training a *17 %* increase in cardiac output to 14 6 l $\times$ min^{-1} was tained ($p < 0\ 01$)

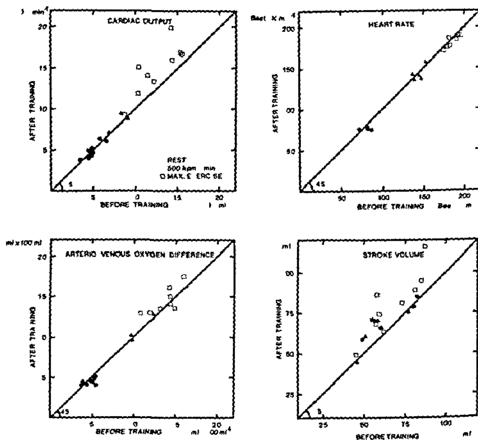


Fig 2 Individual values for cardiac output heart rate arterio venous oxygen difference and stroke volume at rest (supine position) submaximal (500 kpm × min ¹) and maximal exercise

At rest in the supine position before training *stroke volume* (Fig 2 and 4) was 62 ml it decreased by 13 ml in the sitting position ($p < 0.001$) During exercise it increased again and had returned to the level observed during rest in the supine position at a load only of 250 kpm × min ¹ No further increase was noted up to maximal exercise 3 of the boys attained their highest stroke volumes in maximal exercise while the other boys with one exception were within 4 ml of their maximal values Similar results were obtained after training but the level was approximately 20 % higher This time 5 boys attained their greatest stroke volumes in maximal exercise the other 4 were within 4 ml of their highest values While the decrease in cardiac output when changing from the supine to the sitting position was less pronounced after training the decrease in stroke volume was similar

Since there was no difference in oxygen uptake and cardiac output during rest and submaximal exercise *a v oxygen difference (AVD)* was unchanged (Fig 2) At maximal exercise a slightly higher mean value was obtained after training i e 1?7 ml × 100 ml ¹ v 14 2 ml × 100 ml ¹ but this difference was not statistically significant

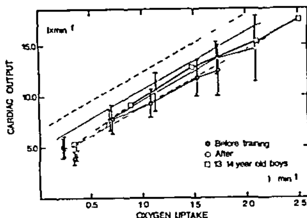


Fig 3 Mean values with ±1 SD of the cardiac output in l×min⁻¹ at rest (supine and sitting positions) and during work including maximal exercise for the 11 7 year old boys before and after the training period Empty squares denote corresponding mean values for 13—14 year old boys (Eriksson Grimby and Saltin 1971) Continuous line and lines with short dashes denote regression line of cardiac output with ±1 SD for young adult men (Ekblom *et al* 1968)

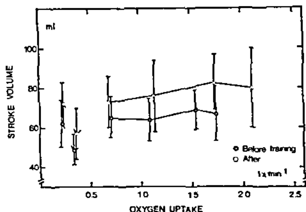


Fig 4 Mean values with ±1 SD of the stroke volume in ml for the 11 7 year old boys at rest (supine and sitting positions) and during work including maximal exercise before and after the training period

Blood lactate concentration at rest and at 500 kpm×min⁻¹ was 1—2 mM and no significant change occurred with training The same was true at maximal exercise during which mean values were around 6 mM

In the pretraining study *mean blood pressure* at rest was 87 mm Hg It gradually increased with increasing work loads reaching 105 mm Hg in maximal exercise A slightly more pronounced pressure response with a difference of 10 mm Hg in maximal exercise ($p < 0.05$) was obtained after training

Neither cardiac output nor mean blood pressure changed at rest and at sub maximal work loads after training Thus *total peripheral resistance (TPR)* (Fig 5) was unchanged Training resulted in an increased maximal cardiac output but TPR was unchanged as mean blood pressure also increased at maximal exercise

Comments

A somewhat lower oxygen uptake was found in maximal work for most of the boys (range 0—0 2 l×min⁻¹ mean 0 1 l×min⁻¹) during the hemodynamic study as compared to the initial study both before and after training The same discrepancy

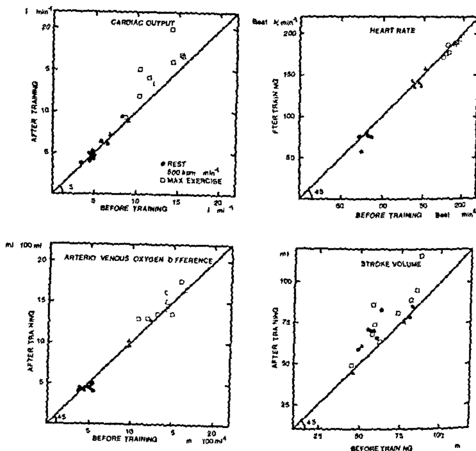


Fig 2 Individual values for cardiac output heart rate arterio venous oxygen difference and stroke volume at rest (supine position) submaximal (500 kpm×min⁻¹) and maximal exercise

At rest in the supine position before training *stroke volume* (Fig 2 and 4) was 62 ml it decreased by 13 ml in the sitting position ($p < 0.001$) During exercise it increased again and had returned to the level observed during rest in the supine position at a load only of 250 kpm×min⁻¹ No further increase was noted up to maximal exercise 3 of the boys attained their highest stroke volumes in maximal exercise while the other boys with one exception were within 4 ml of their maximal values Similar results were obtained after training but the level was approximately 20 % higher This time 5 boys attained their greatest stroke volumes in maximal exercise the other 4 were within 4 ml of their highest values While the decrease in cardiac output when changing from the supine to the sitting position was less pronounced after training the decrease in stroke volume was similar

Since there was no difference in oxygen uptake and cardiac output during rest and submaximal exercise *a v oxygen difference (AVD)* was unchanged (Fig 2) At maximal exercise a slightly higher mean value was obtained after training 14.7 ml×100 ml⁻¹ (14.2 ml×100 ml⁻¹) but this difference was not statistically significant

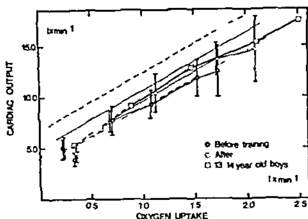


Fig 3 Mean values with ± 1 SD of the cardiac output in l × min⁻¹ at rest (supine and sitting positions) and during work including maximal exercise for the 11 7 year old boys before and after the training period Empty squares denote corresponding mean values for 13—14 year old boys (Eriksson Grimby and Saltin 1971) Continuous line and lines with short dashes denote regression line of cardiac output with ± 1 SD for *young adult men (Ekblom et al* 1968)

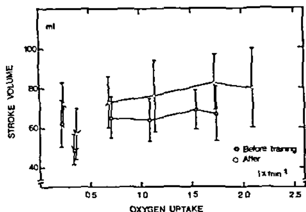


Fig 4 Mean values with ± 1 SD of the stroke volume in ml for the 11 7 year old boys at rest (supine and sitting positions) and during work including maximal exercise before and after the training period

Blood lactate concentration at rest and at 500 kpm × min⁻¹ was 1—2 mM and no significant change occurred with training The same was true at maximal exercise during which mean values were around 6 mM

In the pretraining study *mean blood pressure* at rest was 87 mm Hg It gradually increased with increasing work loads reaching 105 mm Hg in maximal exercise A slightly more pronounced pressure response with a difference of 10 mm Hg in maximal exercise ($p < 0.05$) was obtained after training

Neither cardiac output nor mean blood pressure changed at rest and at submaximal work loads after training Thus *total peripheral resistance (TPR)* (Fig 5) was unchanged Training resulted in an increased maximal cardiac output but TPR was unchanged as mean blood pressure also increased at maximal exercise

Comments

A somewhat lower oxygen uptake was found in maximal work for most of the (range 0—0 2 l × min⁻¹ mean 0 1 l × min⁻¹) during the hemodynamic stu compared to the initial study both before and after training The same d

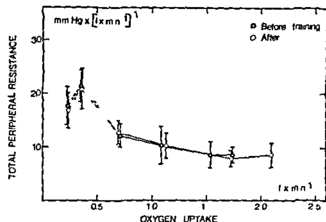


Fig 5 Mean values with ±1 SD of the total peripheral resistance in mm Hg×l⁻¹×min at rest (supine and sitting positions) and during work including maximal exercise for the 11 7 year old boys before and after the training period

has been observed in previous studies (Saltin *et al* 1968 Ekblom *et al* 1968) One explanation for the somewhat lower maximal oxygen uptake value during the hemo dynamic studies could be that the boys were more inhibited by the examination situ ation However it is noteworthy that the differences between oxygen uptakes at maximal exercise in the precatheterization and hemodynamic studies were similar before and after training Therefore the same improvement by training is obtained irrespective of the value used for comparison

Discussion

Previous studies (*cf* Åstrand and Rodahl 1970 pp 378—380) have shown that the effect of training with respect to aerobic power is influenced by factors such as the subjects age sex and initial maximal oxygen uptake as well as training duration and intensity Therefore, it is difficult to compare different studies regarding the effect of training Wide individual variations in improvement *e g* from 1—2 up to 100 per cent have been reported (Daniels and Oldridge 1971 Saltin *et al* 1968) How ever a mean improvement of 10—20 % was found in most studies The boys examined in the present study did not differ from subjects in previous examinations with respect to variation or mean improvement obtained In a previous study of 11 years old boys (Ekblom 1969 a) a 6 months training period produced an increase in maximal oxygen uptake of 15 % or 10 % when body weight was taken into ac count However an additional 26 months of training produced no further increase in oxygen uptake per kg b w On the other hand, heart volume and vital capacity increased more than might me expected from growth

Studies of male adults (Rowell 1962 Saltin *et al* 1968 Ekblom *et al* 1968 Saltin *et al* 1969) have shown an increase in maximal oxygen uptake of the same magni tude This is also the case when initial values for maximal aerobic power are con sidered In the present study the increase in maximal aerobic power is of the same order as that observed in the study of 11 year old boys (Ekblom 1969 a) and in the aforementioned studies of young male adults The increase in stroke volume (19 %)

was slightly higher than the increase in cardiac output implying a slightly lower heart rate (2 beats × min^{-1}) in maximal exercise This response by the central circulation is in good agreement with that seen after the training of middle aged and elderly men (Hartley *et al* 1969) and women of different ages (Kilbom and Astrand 1971) On the other hand training in young adult men produced an increase in both stroke volume and AVD (Rowell 1962 Saltin *et al* 1968 Ekblom *et al* 1968) This different response might have been due to biological differences or differences in the methodological procedure It has been shown (Hartley and Saltin 1969) that an exercise which exhausts a subject within 5—8 min produces a significantly higher cardiac output than a heavier exercise in which exhaustion is reached within 3—4 min despite unchanged oxygen uptake Since work duration in the present study was identical before and after training this explanation is not very likely The boys response to training was similar to that of middle aged and elderly men in respect to the increase in stroke volume This was also true for heart volume An increase in heart volume of 10 % was obtained in these boys but no increase was noted when the effect of growth was considered The middle aged and elderly men had also completely unchanged heart volumes When comparing heart volume and stroke volume in the present study the increase in stroke volume was found to be more pronounced which means an altered relationship between those two volumes This should be kept in mind when making a comparison between fit and unfit subjects in a cross sectional study of children

It is noteworthy that after training no significant increase was found in the a v oxygen difference during exercise One reason for this may be that children are usually more active than adults and have a higher degree of physical fitness An initial AVD increase as a result of training is a well known phenomenon (Saltin *et al* 1968) but this does not occur in rather fit subjects Moreover the boys had a rather low mean hemoglobin concentration of about 13 g × 100 ml^{-1} which however is normal for this age group This gives a lower oxygen binding capacity of their blood The AVD might therefore have been at the maximal attainable level already in the pretraining study Since AVD failed to increase the increase in maximal aerobic power obtained was exclusively due to the rise in cardiac output In the study of 11 year old boys (Ekblom 1969 a) the increase in maximal oxygen uptake appears to be fully explainable by the increase in stroke volume as indicated by the observed increase in heart volume after 32 months of training It is also most likely that no increase in a v oxygen difference was obtained in that study either If an increase in AVD had been obtained however the conclusion reached by Ekblom (1969 b) that rational prepubertal training yields the best result would have been true

At submaximal work loads a greater a v oxygen difference was noted in the present group of boys than in adults (Ekblom *et al* 1968) Similar results were obtained in pubertal boys (Eriksson Grimby and Saltin 1971) The most probable explanation for this circumstance is a different pattern of the blood flow distribution The proportion of flow directed to non working organs might be smaller in children resulting in a lower cardiac output as compared to adults

At submaximal work loads training resulted in a lower heart rate This effect of physical training has been known for many years As early as 1931 (Christensen 1931) it was pointed out that the factors involved in the regulation of the heart rate response during exercise are not well understood It is then difficult to evaluate which mechanisms that are brought into play explaining the lowering of the heart rate observed after training As cardiac output was unaltered at submaximal loads the calculated stroke volume was greater The question then arises as to whether the primary effect of training is an increased stroke volume or a decrease in heart rate Thus Clausen Trap Jensen and Lassen (1970) have published data pointing to a mechanism for extracardial regulation of the sinus node as the cause of heart rate decrease after training This should result in a compensatory increase in the stroke volume The discovery that training did not result in any decrease in the heart rate of vagotomized mice in submaximal exercise (Tipton Barnard and Tcheng 1969) suggests that parasympathetic control is involved This should lend support to the view that physical training primarily affects heart rate On the other hand the increase in stroke volume at maximal exercise found in this study was responsible for the increased maximal cardiac output Before training no difference in stroke volume was found between submaximal and maximal exercise This was also true after training and supports the theory that a stroke volume increase in submaximal exercise is the primary effect of training

A somewhat lower blood pressure was found for the boys than for adults On the other hand total peripheral resistance was of the same order Thus the lower mean blood pressure was probably due to a relatively smaller cardiac output Higher values for pressures and TPR are found in older subjects (Hartley *et al* 1969) but the higher TPR in these subjects may be due to vascular changes caused by aging Training of these middle aged and older men resulted in a slight yet insignificant decrease in blood pressure and TPR

The circulatory response of children to physical training is slightly different from that of adults since there is no increase in the a v oxygen difference at maximal exercise More striking however is the great similarity in reaction Thus the oxygen uptake—cardiac output ratio was identical and stroke volume responded in a similar manner Thus a 4 month training period in prepubertal boys does not appear to improve maximal aerobic power more than corresponding training in young adults

This work was in part supported by grants from the Swedish Delegation for Applied Medical Defence The authors acknowledge the valuable help from Bo Ollander and Inga Lena Runson who designed and supervised the physical training

References

ÅSTRAND P O I ENGSTROM B O ERIKSSON P KARLBERG I NYLANDER B SALTIN and C THOREN Girl swimmers *Acta paediat* (Uppsala) 1963 Suppl 147

ÅSTRAND P O and K RODAHL *Textbook of work physiology* New York Mc Graw Hill Book Company 1970

BEVEGÅRD S Studies on the regulation of the circulation in man With special reference to the stroke volume and the effect of muscular work body position and artificially induced variations of the heart rate *Acta physiol scand* 1962 *52* Suppl 200

BROMAN B G DAHLBERG and A LICHTENSTEIN Height and weight during growth *Acta paediat* (Uppsala) 1942 *30* 1—66

CHRISTENSEN E H Beitrage zur Physiologie schwerer Korperlicher Arbeit *Arbeitsphysiologie II und I* 1931 453—481

CLAUSEN J P J TRAP JENSEN and N A LASSEN The effects of training on the heart rate during arm and leg exercise *Scand J clin Lab Invest* 1970 *26* 295—301

DANIELS J and N OLDRIDGE Changes in oxygen consumption of young boys during growth and running training *Med sci Sports* 1971 *3* 161—165

VON DOBELN W and B O ERIKSSON Physical training maximal oxygen uptake and dimensions of the oxygen transporting and metabolizing organs in boys 11—13 years of age *Acta paediat scand* (Uppsala) 1972 *61* 653—660

EKBLOM B Effect of physical training in adolescent boys *J appl Physiol* 1969 a. *27* 350—355

EKBLOM B Effect of physical training on oxygen transport system in man *Acta physiol scand* 1969 b Suppl 328

EKBLOM B P O ÅSTRAND B SALTIN J STENBERG and B WALLSTROM Effect of training on circulatory response to exercise *J appl Physiol* 1968 *24* 518—528

ERIKSSON B O P D GOLLNICK and B SALTIN Muscle metabolism and enzyme activities after training in boys 11—13 years old *Acta physiol scand* 1973 In press

ERIKSSON B O G GRIMBY and B SALTIN Cardiac output and arterial blood gases during exercise in pubertal boys *J appl Physiol* 1971 *31* 348—352

HAMILTON W F R L RILEY A M ATTYAH A COURNAND D M FOWELL A HIMMELSTEIN R P NOBLE J W REMINGTON D W RICHARDS JR N C WHEELER and A C WITHAM Comparison of Fick and dye injection methods of measuring cardiac output in man *Amer J Physiol* 1948 *153* 309—321

HARTLEY L H and B SALTIN Blood gas tensions and pH in brachial artery femoral vein and brachial vein during maximal exercise *Medicine and Sport* 1969 *3* 66—72

HARTLEY L H G GRIMBY A KILBOM N J NILSSON I ÅSTRAND J BJURE B EKBLOM and B SALTIN Physical training in sedentary middle aged and older men III Cardiac output and gas exchange at submaximal and maximal exercise *Scand J clin Lab Invest* 1969 *24* 335—344

KILBOM A and I ÅSTRAND Physical training with submaximal intensities in women B Effect on cardiac output *Scand J clin Lab Invest* 1971 *28* 163—175

KJELLBERG S R U RUDHE and T SJOSTRAND The relation of cardiac volume to the weight and surface of the body the blood volume and the physical capacity for work *Acta radiol* (Stockh) 1949 *31* 113—122

KOCH G and B O ERIKSSON Ventilation and intrapulmonary gas exchange at rest and during submaximal and maximal work in 11—13 year-old boys before and after physical training *Scand J clin Lab Invest* 1972 a In press

KOCH G and B O ERIKSSON Effect of physical training on anatomical R L shunt at rest and pulmonary diffusing capacity during near maximal exercise in boys 11—13 years old *Scand J clin Lab Invest* 1972 b In press

ROWELL L B *Factors affecting the prediction of maximal oxygen intake from measurements during submaximal work with observations related to factors which may limit maximal oxygen uptake* Mineapolis Univ of Minnesota. 1962

SALTIN B Oxygen transport by the circulatory system during exercise in man *Proc Symposium on Limiting Factors of Physical Performance* Ed J Keul Freiburg 1972

SALTIN B L H HARTLEY A KILBOM and I ÅSTRAND Physical training in sedentary middle aged and older men II Oxygen uptake heart rate and blood lactate concentration at submaximal and maximal exercise *Scand J clin Lab Invest* 1969 *24* 323—334

SALTIN B C G BLOMQVIST J H MITCHELL R L JOHNSON K WILDENTHAL and C B CHAPMAN Response to exercise after bed rest and after training *Circulation* 1968 *38* Suppl 7

SCHOLZ R H SCHMITZ T BUECHER and J O LAMPEN Über die Wirkung von Nystatin auf Backerhefe *Biochem Z* 1959 *331* 71—86

SCHWARTZ L R H BRITTEN and L R THOMPSON Studies in physical development and posture I The effect of exercise on the physical condition and development of adolescent boys *Publ Hlth Bull* (Wash) 1928 *179*

SJOSTRAND T A A method for the determination of the total hemoglobin content of the body *Acta physiol scand* 1948 *16* 211—231

TIPTON C M R J BARNARD and T K TCHENG Resting heart rate investigations with trained and untrained hypophysectomized rats *J appl Physiol* 1969 *26* 585—588

Acta physiol scand 1973 87 40—56

From the Nobel Institute for Neurophysiology, Karolinska Institutet Stockholm Sweden

Repetitive Impulse Firing Comparisons between Neurone Models Based on 'Voltage Clamp Equations' and Spinal Motoneurones

By

Daniel Kernell and Hans Sjoholm

Received 10 April 1972

Abstract

Kernell D and H Sjoholm *Repetitive impulse firing comparisons between neurone models based on voltage clamp equations and spinal motoneurones* Acta physiol scand 1973 87 40—56

Repetitive impulse firing was elicited in neurone models by steady stimulating currents of abrupt onset The neurone models were based on the Frankenhaeuser—Huxley equations (1964) for voltage clamp data from the amphibian peripheral nerve A frequency current curve (f I curve) initial adaptation and minimum firing rate similar to those of cat spinal motoneurones were obtained in the Frankenhaeuser—Huxley model if it were provided with (i) a long lasting after hyperpolarization due to potassium permeability changes and (ii) a decreased subthreshold sodium inactivation For detailed comparisons to the repetitive impulse firing of spinal motoneurones model versions were used in which the subthreshold sodium inactivation was very slight and the passive membrane properties as well as the afterpotentials resembled those of spinal motoneurones In their repetitive behaviour these models were quantitatively similar to spinal motoneurones In the motoneurone like model versions initial adaptation was due to a kind of summation of the potassium permeability changes underlying the after hyperpolarizations of consecutive spikes The slope of the f I curve was markedly affected by modifications of the size or time course of the potassium permeability changes responsible for the after hyperpolarization

For the understanding of the integrative properties of the central nervous system it is important to know how the firing rate of single neurones may be controlled by maintained currents such as those produced by asynchronous synaptic activity We have used mathematical neurone models (i e models consisting of equation systems) in order to study the probable importance of various membrane properties for the kind of repetitive impulse firing that is elicited in spinal motoneurones by steady current Our neurone models (Kernell and Sjoholm 1972) were based on the Frankenhaeuser—Huxley equations (1964) for Frankenhaeuser's voltage clamp data from the amphibian myelinated nerve It is known that the original Frankenhaeuser—Huxley model (F—H model) may fire repetitively in response to a steady stimulating current (Frankenhaeuser and Vallbo 1965 Bromm 1969 Bromm and Frankenhaeuser 1972) We have modified the original F—H model in various ways

in order to make some of its membrane properties similar to those of spinal moto neurones The motivations for and the nature of these various modifications were described in a preceding paper (Kernell and Sjoholm 1972) In the present paper the repetitive impulse firing of these different model versions will be compared to that of spinal motoneurons A brief summary of earlier experimental findings concerning repetitive firing in motoneurones is given below Preliminary reports of the present findings have been published (Kernell and Sjoholm 1971 a b)

Repetitive impulse firing in cat spinal motoneurones In cat spinal motoneurones repetitive impulse firing has to a great extent been studied with the aid of maintained stimulating currents that were injected into single cells via a microelectrode These studies have shown that the curve relating firing rate to the intensity of steady stimulating current (the f I curve) is approximately linear over a range of lower firing rates (primary range) (Granit Kernell and Shortess 1963 a Kernell 1965 a b) At higher firing rates produced by stronger currents the slope of the f I curve becomes steeper (secondary range) (Kernell 1965 b Granit Kernell and Lamarre 1966) Motoneurones are not always capable of maintained firing within the secondary range all motoneurones are however capable of firing within the secondary range for brief initial periods after the abrupt onset of stimulation (Kernell 1965 b Baldissera and Gustafsson 1971 a) Just after the abrupt onset of a steady stimulating current producing a maintained discharge there is an initial decrease of firing rate (initial adaptation) which is associated with a decrease in the slope of the f I curve (f I slope Granit *et al* 1963 a Kernell 1965 a b) For brief periods steady stimulating current may produce firing rates as high as 300—600 imp/s (Kernell 1965 b) In most lumbrasacral motoneurones regular firing may be main tained at rates as low as 20 imp/s or less (Kernell 1965 c) Such a wide range of firing rates may also be obtained by synaptic activation of spinal motoneurones (*e g* Adrian and Bronk 1929 Wilson and Burgess 1962)

Methods

The modified versions of the F H model that are used in the present investigation were developed and described in detail in a preceding paper (Kernell and Sjoholm 1972) Nomen clature symbols equations data and methods of computation were also given in the preceding paper (Kernell and Sjoholm 1972) In the present paper repetitive impulse firing was elicited in the neurone models by steady transmembrane currents of stepwise onset Impulse intervals were measured between spike peaks In illustrations the term steady firing refers to portions of a discharge where 2 consecutive impulse intervals differed by less than 1 % In many of these cases the consecutive intervals and spikes were virtually identical Prior to the onset of stimulation membrane potential was always at the resting level (—70 mV)

In model No 2 small subthreshold oscillations might occur just prior to the spikes following the first impulse at low stimulus intensity (oscillations of about 0 6 mV or less seen at 12 nA stimulation for the model version used in Fig 4 A—B) In some varieties of model No 1 one or several negative going dips (up to about 2 5 mV in amplitude due to activation of potassium permeability) occurred just prior to the second and later spikes in discharges elicited by weak stimuli (seen at 8 nA for model version used in Fig 5 and at 22 and 26 nA for model version used in Fig 11) Discharges containing such prespike dips were not included in illustrated diagrams of f I relations (Fig 5 and 11) It was checked that the prespike oscillations and dips described above were not the result of too long integration steps

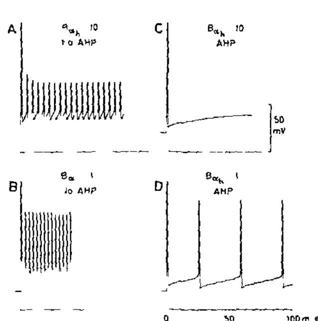


Fig 1 Computed impulse discharges (plots of membrane potential versus time) for models stimulated by steady current of 0 7 mA/cm Abrupt start of stimulation at the onset of the horizontal bar (with time marks) under each potential time curve Resting membrane potential prior to the onset of stimulation Stimulating current lasted to the end of each plot Same potential and time calibration for A—D A Standard Frankenhaeuser Huxley model (F—H model) B F—H model with B_h = −1 mV C F—H model with standard value of B_h (−10 mV) but with slow potassium permeability (P_k) with data as in Fig 7 G of Kernell and Sjoholm (1972) except that P_{K_s} was 4×10 cm/s 1_q was 0 01 ms^{-1} mV^{-1} and β_q was 0 00004 ms^{-1} mV^{-1} D F—H model with B_h = −1 mV and with the same slow potassium system as in C

For comparison to the models some experimental results from cat lumbosacral motoneurones are quoted in the text These results were obtained by conventional techniques for intracellular stimulation and recording (cf Kernell 1965 a) The single barrelled microelectrodes were generally filled with 2 M potassium citrate The cells were stimulated by steady current injected via the recording microelectrode The cats were generally anesthetized by Nembutal

Results

Standard F H model Fig 1 A shows an example of repetitive impulse firing elicited by steady current in the standard model of Frankenhaeuser and Huxley (1964) In this model version the rheobase was much lower than the threshold current for repetitive impulse firing (cf Bromm and Frankenhaeuser 1972) Over a narrow range of weak currents a steady stimulus would produce only 2—3 spikes but not steady firing In maintained discharges produced by slightly stronger currents there was however practically no initial decrease of firing rate (Fig 1 A) If the impulse intervals of such maintained discharges were measured between spike peaks then there was actually some increase of firing rate from the first to the second impulse interval particularly at stronger current intensities (cf Bromm and Frankenhaeuser 1972) The initial spike was however markedly larger than the subsequent ones (Fig 1 A) No such pronounced change of firing rate from the first to the second interval was observed if these impulse intervals were measured between points low down on the falling limbs of the spikes (e g at 20 mV above resting potential) instead of between the spike peaks Firing at rates below 100 imp/s could not be elicited in the standard F H model by steady current The f I curve was convex upwards The f I relation for the standard F H model has recently been described by others (Bromm and Frankenhaeuser 1972)

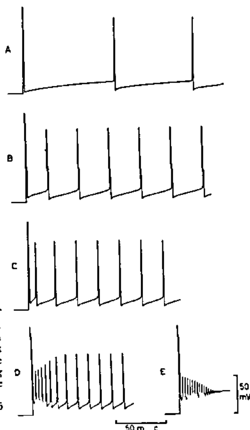


Fig 2 Computed impulse discharges (plots of membrane potential versus time) for model version of Fig 1 D Resting membrane potential prior to the abrupt onset of stimulating current in each case (onset of stimulation just before first spike of each discharge) Stimulating current lasted to the end of each plot Same potential and time calibration for A–E Stimulus intensities 0 45 mA/cm (A) 0 75 mA/cm (B) 1 00 mA/cm (C) 1 60 mA/cm (D) and 1 80 mA/cm (E)

The standard F H model behaved markedly different from spinal motoneurones (*cf* p 41) but relatively similar to the Hodkin Huxley model for the giant axon of the squid (Agin 1964 Stein 1967) with respect to the lack of initial adaptation the high minimum firing rate and the upward convex shape of the f I curve

Standard F H model at 38° C In the standard F H model temperature was shifted from 20° C to 38° C with the aid of the temperature coefficients for rate constants (αs and β's) and permeability constants ($\overline{P}_{Na}$ $\overline{P}_{p}$ P'_{K}) used by Frankenhaeuser (1963 Frankenhaeuser and Moore 1963) Temperature was also of course changed in the constant field equation The firing rates were much higher and the slope of the f I curve was steeper at 38° C than at 20° C The general shape of the f I curve was similar at 38° C and at 20° C (convex upwards) There was very little initial adaptation in maintained discharges at 38° C (small adaptation for weak stimuli no adaptation for strong stimuli)

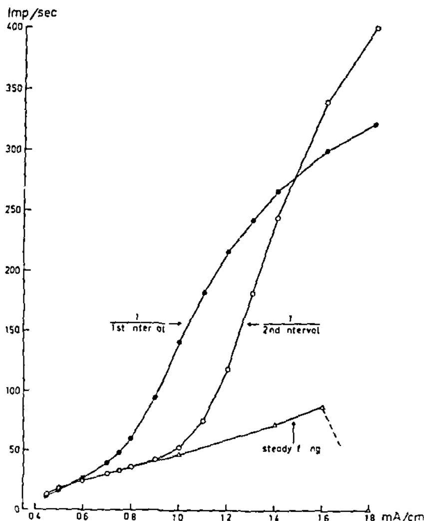


Fig 3 Impulse frequency (imp s) plotted versus stimulus intensity (m \/cm) for the model of Fig 1 D Stimulation by steady currents of abrupt onset (*cf* Fig 1 D and 2) Filled circles values f r first impulse interval after onset of stimulation Open circles values for second impul e nterv il Triangles values for steady firing Plotted values connected by straight lines N firing was elicited by a stimul ition of 0 40 m \/cm

F H model with decreased subthreshold sodium inactivation (B_{a_h} = −1 mV) For the model version of Fig 1 B subthreshold sodium inactivation was decreased by changing B_{a_h} from −10 to −1 mV (Kernell and Sjoholm 1972) In this model version (Fig 1 B) the repetitive spikes were larger and the discharge frequencies elicited by identical currents were somewhat higher than in the standard F H model (Fig 1 A) There was still no adaptation in maintained discharges (Fig 1 B) the f I curve was convex upwards and firing rates below 100 imp/s could not be elicited by steady stimulating currents Particularly for strong stimuli there was an increase of firing rate initially in the discharges (impulse intervals measured

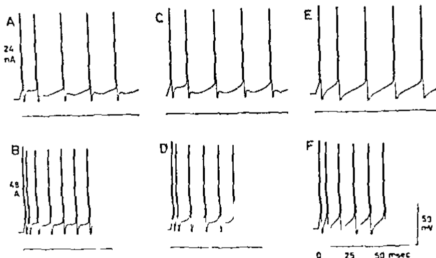


Fig 4 Computed impulse discharges (plots of membrane potential versus time) for motoneurone like model Stimulation by steady current as in Fig 1 Resting membrane potential prior to the abrupt onset of stimulation Same voltage and time calibration for A–F Stimulus intensity 24 nA for A, C and E 48 nA for B D and F A–B Model No 2 C–F Model No 1 For A–F data for the slow potassium permeability (P_{K_s}) and the non specific permeability (P_p) were as in Fig 7 G of Kernell and Sjoholm (1972) with the following exceptions in A–B A_q = 0 0?2 ms^{-1} mV^{-1} and $\bar{P}_p$ = 0 0041 × 10^{-4} cm /s in E–F $\bar{P}_p$ was zero

between spike speaks) Maintained firing could be obtained over a larger range of current intensities in the model of Fig 1 B than in the standard F H model of Fig 1 A (*cf* Stein 1967)

F H model with after hyperpolarization Fig 1 C was obtained from a F H model in which each spike was followed by a long lasting increase of potassium permeability (slow potassium system Kernell and Sjoholm 1972) Thus in this version a single spike elicited by a brief stimulus was succeeded by a prolonged after hyperpolarization From other points of view the model was identical to the standard F H model (thus B_{a_h} = −10 mV) In this model version (Fig 1 C) we obtained no repetitive impulse firing within the duration of our steady stimuli (0 7 0 8 1 0 1 ? 1 4 2 2 and 3 0 mA/cm stimulus intensities applied during 97 71 50 39 36 34 and 114 ms respectively) In the model of Fig 1 C the initial spike was followed by slow subthreshold potential changes The sodium inactivation then had time for a considerable increase If the post spike decay of slow potassium permeability (P_{F_2}) was made faster than that of Fig 1 C an increased tendency for repetitive firing was actually observed

F H model with after hyperpolarization and decreased subthreshold sodium inactivation (B_h = −1 mV) For the model version of Fig 1 D and 2 the modifications of Fig 1 B and C have been combined With these changes the model became capable of firing steadily at rates below 20 imp/s and the f I curve for the first and

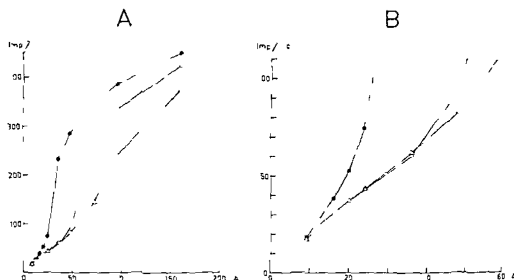


Fig 5 A—B Frequency current curve for model version of Fig 4 C—D Plotted as in Fig 3 Values for firing rates below 100 impulses/sec shown in detail in B No firing was elicited by stimulation of 7 nA In repetitive discharges represented in the diagrams (A—B) the spike peaks were all more than 90 mV positive to the resting membrane potential (i e more than 20 mV overshoot) Atypical adaptation at stimulus intensity 72 nA In this case the first nine impulse intervals were 2 89 3 71 6 05 7 41 6 46 7 12 6 64 6 98 and 6 74 ms Inverse value of the mean of intervals 4—9 plotted as frequency of steady firing at 72 nA

the second impulse intervals (Fig 3) acquired a shape similar to that of spinal motoneurones (primary range with low f I slope at frequencies below about 40 imp/s secondary range with steeper f I slope at higher firing rates) Over a wide range of stimulus intensities there was an evident initial adaptation (Fig 1 D and 2) that was associated with a decrease in slope of the f I curve (Fig 3) For steady firing the f I curve was roughly linear throughout (Fig 3) Thus this particular model version (Fig 1 D 2 3) showed an f I relation rather similar to that of such spinal motoneurones which are uncapable of maintained firing within the secondary range (see Introduction p 41)

It should be noted that the change of $B\alpha_h$ from -10 mV in Fig 1 C to -1 mV in Fig 1 D produced a marked change in the ability for tonic firing without producing any great change in the amplitude of the initial spike (*cf* Frankenhaeuser and Vallbo 1965 see also Fig 1 in Kernell and Sjoholm 1972)

Motoneurone like model versions (Mo 1 Mo 2) Comparisons to spinal motoneurones The impulse discharges in Fig 4 A—D were produced in versions of the motoneurone like models Mo 2 (A—B) and Mo 1 (C—D) (Kernell and Sjoholm 1972) In the model versions of Fig 4 A—D subthreshold sodium inactivation was minimal and the passive membrane properties and the afterpotentials following a single spike were rather similar to those of fast spinal alpha motoneurones (see

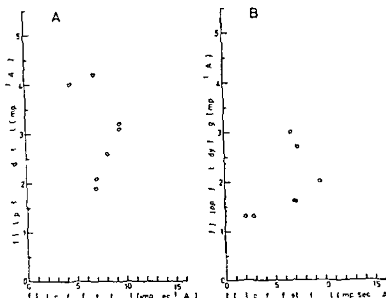


Fig 6 From cat lumbosacral motoneurones Effect of initial adaptation on the slope of the f I curve (f I slope) All values refer to firing within the primary range A Values of the f I slope for the second impulse interval plotted versus that of the first one after the abrupt onset of stimulation (34 different motoneurones) Correlation coefficient +0.78 (t test $P < 0.001$) B Values for the f I slope of steady firing plotted versus that for the first impulse interval after the abrupt onset of stimulation (26 different motoneurones thick walled circles indicate two values with the same coordinates) Correlation coefficient +0.63 (t test $P < 0.001$)

Kernell and Sjoholm 1972 for a full description for the model version of Fig 4 C—D the afterpotentials following a single spike elicited by brief stimulation were shown in Fig 7 G of Kernell and Sjöholm 1972) In order to save computation time only one of the two motoneurone like model versions (Mo 1) was used for detailed studies of repetitive firing Model Mo 1 was chosen because in comparison to this model version (Fig 4 C—D) the illustrated version of model Mo 2 (Fig 4 A—B) had the disadvantage that it tended to run into relatively prolonged sub threshold oscillations at weak stimulus intensity (see p 41) Furthermore in comparison to model Mo 2 model Mo 1 was somewhat closer to the original F H model (see Fig 6 in Kernell and Sjoholm 1972)

The f I relation for the model version of Fig 4 C—D (Mo 1) is shown in Fig 5 Initially as well as for steady firing the slope of the f I curve was less steep at low firing rates (*e g* between 20—50 imp/s) than at higher ones (*e g* between 100—200 imp/s) Thus there was something like a primary and a secondary range (*cf* p 41) In Fig 5 the primary range characterized by a low slope of the f I curve (Kernell 1965 b) appeared to extend from about 17—18 imp/s up to roughly 53 (first interval) 60 (second interval) and 83 (steady firing) imp/s These values are similar to those for lumbosacral motoneurones with a relatively brief after hy

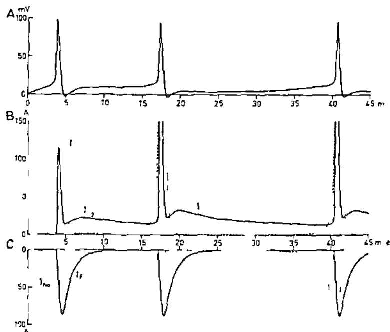


Fig 7 Membrane potential (A) and ionic currents (B—C) plotted versus time for repetitive impulse discharge elicited by steady stimulating current of 24 nA in the model of Fig 4 C—D Abrupt onset of steady stimulating current at time zero During spikes potassium (I_K for 2 last spikes I_K) and sodium (I_N) currents shoot outside the diagrams In this and subsequent illustrations membrane potential is given relative to resting membrane potential

polarization (Kernell 1965 c) however in the latter case the maximum firing rate within the primary range often tends to be about the same initially as later on (Kernell 1965 b) In Fig 5 there was clearly an adaptation Over a considerable range of weaker currents this adaptation was associated with a decrease in the slope of the f I curve (Fig 5) For the roughly linear portion of the f I curve within the primary range the slope was about 3 0 imp s^{-1}nA^{-1} for the first impulse interval and approximately 1 6 imp s^{-1}nA^{-1} for the second impulse interval and for steady firing (Fig 5 B) These f I slopes were within the range of values for spinal motoneurones (Fig 6) Within the primary range the ratio between the f I slope for the first interval and that for the second one (i e a measure of the degree of initial adaptation) was similar to the corresponding ratio for spinal motoneurones (Fig 6 see p 52 for further comments) In Fig 5 the f I slope for the first impulse interval was about 4 4 times steeper at firing rates of 74—233 imp/s (within secondary range) than at firing rates of 18—53 imp/s (primary range) This is similar to the corresponding ratio in spinal motoneurones (cf Kernell 1965 b)

The data summarized above show that there were several important similarities between the behaviour of the model version of Fig 5 and the behaviour of spinal motoneurones As might be expected there were also some differences Thus for instance the model of Fig 5 (Mo 1) could fire steadily over a much wider range

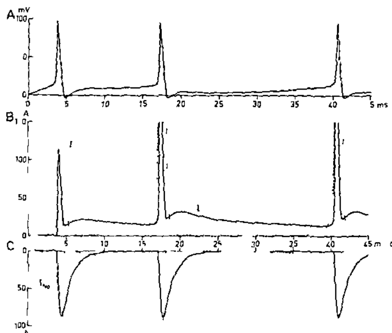


Fig 7 Membrane potential (A) and ionic currents (B – C) plotted versus time for repetitive impulse discharge elicited by steady stimulating current of 24 nA in the model of Fig 4 C – D Abrupt onset of steady stimulating current at time zero During spikes potassium (I_{K_2} for 2 last spikes I_K) and sodium (I_{Na}) currents shoot outside the diagrams In this and subsequent illustrations membrane potential is given relative to resting membrane potential

polarization (Kernell 1965 c) however in the latter case the maximum firing rate within the primary range often tends to be about the same initially as later on (Kernell 1965 b) In Fig 5 there was clearly an adaptation Over a considerable range of weaker currents this adaptation was associated with a decrease in the slope of the f I curve (Fig 5) For the roughly linear portion of the f I curve within the primary range the slope was about 3 0 imp s^{-1}nA^{-1} for the first impulse interval and approximately 1 6 imp s^{-1}nA^{-1} for the second impulse interval and for steady firing (Fig 5 B) These f I slopes were within the range of values for spinal motoneurones (Fig 6) Within the primary range the ratio between the f I slope for the first interval and that for the second one (*i e* a measure of the degree of initial adaptation) was similar to the corresponding ratio for spinal motoneurones (Fig 6 see p 52 for further comments) In Fig 5 the f I slope for the first impulse interval was about 4 4 times steeper at firing rates of 74—233 imp/s (within secondary range) than at firing rates of 18—53 imp/s (primary range) This is similar to the corresponding ratio in spinal motoneurones (*cf* Kernell 1965 b)

The data summarized above show that there were several important similarities between the behaviour of the model version of Fig 5 and the behaviour of spinal motoneurones As might be expected there were also some differences Thus for instance the model of Fig 5 (Mo 1) could fire steadily over a much wider range

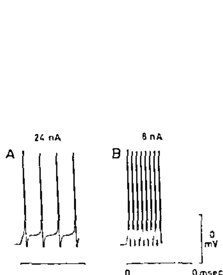


Fig 10

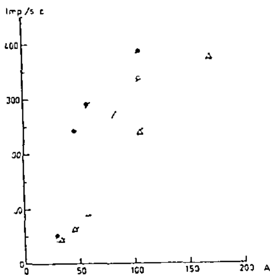


Fig 11

Fig 10 Computed impulse discharges (plots of membrane potential versus time) for model version of Fig 9 B Stimulation by steady current as in Fig 1 Stimulus intensities as indicated Resting membrane potential prior to the abrupt onset of stimulation Same potential and time calibration for A—B

Fig 11 Frequency current plot for model like that of Fig 4 C—D but with leak conductance (g_L) equal to $2\,0 \times 10^{-}$ mho instead of $1\,0 \times 10^{-4}$ mho Symbols as in Fig 3 Interrupted lines show the frequency current curves of Fig 5 shifted 10 nA to the right No repetitive impulse firing was elicited by a stimulus intensity of 19 nA

by the spikes (*cf* Fig 7 I_{K_2}) This summation of the potassium permeability underlying consecutive after hyperpolarizations was responsible for initial adaptation in the models

The diagram of Fig 9 A was from the motoneurone like model version of Fig 5 It shows the after hyperpolarizations succeeding two consecutive spikes Each spike was elicited by a brief stimulus pulse The after hyperpolarization following the second spike was much larger than that following the first spike alone (Fig 9 A) The degree of apparent summation of after hyperpolarizations in Fig 9 A was similar to the degree of summation that may be observed in spinal motoneurones after a pair of antidromic spikes (Ito and Oshima 1962) In the present kind of neurone model it is however not at all self evident that there should be a summation of after hyperpolarizations This is demonstrated by the case of Fig 9 B The size and time course of the after hyperpolarization following a single spike in Fig 9 B was very similar to that in Fig 9 A However for the case shown in Fig 9 B there was virtually no summation of the after hyperpolarizations following two consecutive spikes both after hyperpolarizations have virtually the same peak amplitude In the model version with no summation of after hyperpolarizations (Fig 9 B) there was no initial adaptation in maintained repetitive discharges elicited by steady current (Fig 10) The f I curve for the non adapting model version of

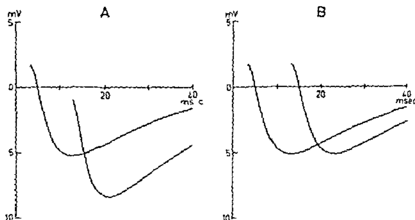


Fig 9 After hyperpolarizations following two consecutive spikes in model version of Fig 4 C–D (A) and in model version like that of Fig 4 C–D but with P_K = 0 0111 × 10 cm /s and f_c = 0 4 ms⁻ mV⁻ (B) The plots show only the portions of the afterpotentials that follow the peak of the delayed depolarization (*cf* Kernell and Sjoholm 1972) thus the spikes themselves are not shown In each case (A and B) one brief rectangular stimulus pulse was given at time zero and a second one 10 ms later Each stimulus was 1000 nA and it lasted in each case until the membrane potential had reached a value 18 mV positive to the resting potential Stimulus duration was about 0 09 ms for the first spike and 0 12 ms for the second spike in A as well as in B Each pulse elicited only one spike In A as well as in B the afterpotential following the first spike is shown as it was in absence of stimulation 10 ms later

The effect on the f I slope of changes in the time course of the permeability changes underlying the after hyperpolarization was not tested in the model version of Fig 5 In the model version of Fig 2—3 it was noted however that the slope of the f I curve within the primary range became increased by 80 % or more if the rate of decay of P_{K_2} was doubled (produced by doubling $A\beta_q$ *cf* Kernell and Sjoholm 1972)

In the motoneurone like model version of Fig 5 the non specific permeability (P_p) was responsible for the delayed depolarization after a single spike (Kernell and Sjoholm 1972) In the repetitive discharges of this model P_p (and I_p) was of some importance at high firing rates If P_p was zero throughout in the model version of Fig 5 then the currents of Fig 5 still produced about the same impulse frequencies within the primary range but somewhat lower frequencies within the secondary range (*cf* Fig 4 D and F)

Adaptation and the summation of after hyperpolarizations In the present model versions (Fig 2 and 4) the occurrence of initial adaptation was dependent on the presence of a slow potassium system (P_{K_2}) If P_{K_2} was zero throughout then there was no adaptation (*cf* Fig 1 B and D)

During initial adaptation in the models there was a progressive growth in the size of successive after hyperpolarizations (Fig 2 and 4) Similar phenomena have been observed in spinal motoneurones (Granit *et al* 1963 a) In the present models the growth in size of consecutive after hyperpolarizations was due to a progressive increase in the extent to which slow potassium permeability (P_{K_2}) was activated

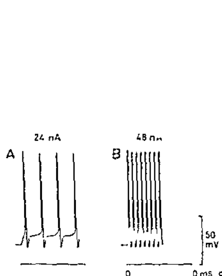


Fig 10

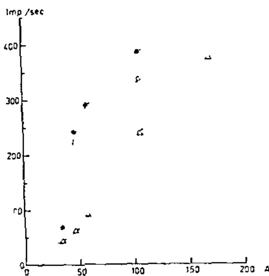


Fig 11

Fig 10 Computed impulse discharges (plots of membrane potential versus time) for model version of Fig 9 B Stimulation by steady current as in Fig 1 Stimulus intensities as indicated Resting membrane potential prior to the abrupt onset of stimulation Same potential and time calibration for A–B

Fig 11 Frequency current plot for model like that of Fig 4 C–D but with leak conductance (g_L) equal to 2.0×10^{-4} mho instead of $1.0 \times 10^{-}$ mho Symbols as in Fig 3 Interrupted lines show the frequency current curves of Fig 5 shifted 10 nA to the right No repetitive impulse firing was elicited by a stimulus intensity of 19 nA

by the spikes (*cf* Fig 7 I_{K_2}) This summation of the potassium permeability underlying consecutive after hyperpolarizations was responsible for initial adaptation in the models

The diagram of Fig 9 A was from the motoneurone like model version of Fig 5 It shows the after hyperpolarizations succeeding two consecutive spikes Each spike was elicited by a brief stimulus pulse The after hyperpolarization following the second spike was much larger than that following the first spike alone (Fig 9 A) The degree of apparent summation of after hyperpolarizations in Fig 9 A was similar to the degree of summation that may be observed in spinal motoneurones after a pair of antidromic spikes (Ito and Oshima 1962) In the present kind of neurone model it is however not at all self evident that there should be a summation of after hyperpolarizations This is demonstrated by the case of Fig 9 B The size and time course of the after hyperpolarization following a single spike in Fig 9 B was very similar to that in Fig 9 A However for the case shown in Fig 9 B there was virtually no summation of the after hyperpolarizations following two consecutive spikes both after hyperpolarizations have virtually the same peak amplitude In the model version with no summation of after hyperpolarizations (Fig 9 B) there was no initial adaptation in maintained repetitive discharges elicit by steady current (Fig 10) The f I curve for the non adapting model ve

Fig 9 B and 10 was very close to the f I curve for the first impulse interval in Fig 5

The different degree of summation of after hyperpolarizations in Fig 9 A and B was due to differences in the potential and time dependence of the slow potassium permeability (P_{K_s} dependent on variable q Kernell and Sjoholm 1972) For the case of Fig 9 B the potential and time dependent variable q reached a value of maximally 0 99 during a single spike Thus in this case q reached a value close to its maximum possible value of 1 00 already during the first spike of a discharge Consequently there could be hardly any summation of after hyper polarizations (Fig 9 B) and no initial adaptation (Fig 10) For the model version of Fig 9 A the variable q reached a peak value of only about 0 46 during a single spike Consequently higher peak values of q could be obtained during subsequent spikes and a summation of after hyperpolarizations (Fig 9 A) and an initial adaptation (Fig 5) could occur The degree of initial adaptation and the degree of summation of after hyperpolarizations could be varied over a wide range by changes in the constant A_{α_q} (see Kernell and Sjoholm 1972)

In maintained discharges of the present models adaptation was generally largely over after one (at weak stimuli) or a few (at stronger stimuli) initial impulse intervals (Fig 2 and 4) Within the primary range there was no further decrease of f I slope beyond the second impulse interval (Fig 3 5 and 8) In spinal motoneurones firing within the primary range most of the initial decrease in f I slope typically occurs from the first to the second impulse interval but there is usually some further decrease of f I slope beyond the second impulse interval (ratio between f I slopes for first and second interval $2\ 20 \pm 0\ 16$ ratio between f I slopes for second interval and steady firing $1\ 36 \pm 0\ 06$ mean $\pm$ SE $n = 25$ motoneurones) In some spinal motoneurones a gradual decrease of firing rate associated with a decrease in f I slope might continue for as long as 1 s of steady stimulation (Kernell 1965 a) Furthermore in spinal motoneurones there may also be a *late adaptation* that is not associated with a change in f I slope (Granit *et al* 1963 b Kernell 1965 a) Such prolonged periods of adaptation in spinal motoneurones should be due to long time-constant processes that were not included in the present model versions

The effect of maintained changes in membrane conductance (leak conductance) In spinal motoneurones maintained post synaptic conductance changes of considerable magnitude may occur without affecting the f I slope for steady firing within the primary range (Kernell 1969) Therefore it was of interest to see how the f I relation of a motoneurone like model version would be affected by a maintained conductance change

The model version of Fig 11 corresponds to that of Fig 5 except for the fact that the cellular leak conductance (g_L) was $1\ 0 \times 10^{-6}$ mho for Fig 5 and $2\ 0 \times 10^{-6}$ mho for Fig 11 This halving of the resting input resistance resulted as expected in an increased threshold current for repetitive firing For the stimulus intensities of Fig 11 the slope and shape of the f I curve were however not significantly changed Interrupted lines in Fig 11 show the f I curves of Fig 5 shifted 10 nA to the right

Discussion

The modifications of the F H model that were used in the present paper were made in order to imitate certain properties of single spike discharges in motoneurones (see Introduction of Kernell and Sjoholm 1972) Thus the design of these modifications was *not* directly guided by the effects of the modifications on the repetitive impulse firing in the models Therefore the many similarities between the repetitive behaviour of the present motoneurone like models (*e g* Fig 4—5) and spinal motoneurones (*e g* Kernell 1965 a b c) suggest that the models actually may incorporate major mechanisms of the repetitive impulse firing of spinal motoneurones Furthermore it is interesting to note that many of the repetitive properties of spinal motoneurones were acquired by a model for peripheral nerve (standard F H model) if the latter was provided with (i) a long lasting after hyperpolarization due to potassium permeability changes and (ii) a decreased subthreshold sodium inactivation (Fig 1—3) The present model studies tend to support the view that the properties of repetitive firing in motoneurones (*e g* shape and slope of f I curve initial adaptation ability to fire at slow rates in response to steady stimulation) are to a great extent dependent upon the properties of their prolonged after hyperpolarization (*cf* also Eccles 1936 1953 Pitts 1943 Eccles Eccles and Lundberg 1958 Kernell 1965 c 1968 Baldissera and Gustafsson 1971 b) Besides, to make maintained firing in response to steady stimulation at all possible (particularly at slow discharge rates) it is apparently important that the subthreshold sodium inactivation is not too great (Fig 1)

The neurone models of the present paper are complex and the extensive calculations involved require a computer A much simpler mathematical model has recently been used for interpreting certain aspects of repetitive impulse firing in motoneurones (Kernell 1968 1970) In this simple model a spike was assumed to occur as soon as a fixed threshold potential was reached Each spike was associated with a sharp rise in potassium conductance which slowly decayed in the post spike period Capacity currents were neglected If the post spike decline of potassium conductance was exponential the f I curve showed an approximately linear region corresponding to the primary range and the slope of the f I curve became steeper for impulse frequencies and steady stimulating currents exceeding those within the primary range (Kernell 1968) In such a model it may be shown that the f I slope within the primary range is inversely proportional to (i) the time constant of the post spike decay of potassium conductance (ii) the size of the post spike change of potassium conductance (as measured at a given time for a given rate of decay) and (iii) the difference between the threshold potential and the equilibrium potential for potassium (*cf* Kernell 1968) Thus the dependence of the f I slope on the size and time course of the events underlying the after hyperpolarization was similar in this simple model and in the complex models discussed in the present paper (p 49—50 *cf* Fig 5 and 8) Furthermore neither in the simple model (Kernell 1968) nor in the complex models of Fig 5 and 11 was the f I slope markedly affected by steady conductance changes One notable limitation of the simple model (Kernell 19

Fig 9 B and 10 was very close to the f I curve for the first impulse interval in Fig :

The different degree of summation of after hyperpolarizations in Fig 9 A and B was due to differences in the potential and time dependence of the slow potassium permeability (P_{K_s} dependent on variable q, Kernell and Sjoholm 1972) For the case of Fig 9 B the potential and time dependent variable q reached a value of maximally 0 99 during a single spike Thus in this case q reached a value close to its maximum possible value of 1 00 already during the first spike of a discharge Consequently, there could be hardly any summation of after hyperpolarizations (Fig 9 B) and no initial adaptation (Fig 10) For the model version of Fig 9 A the variable q reached a peak value of only about 0 46 during a single spike Consequently higher peak values of q could be obtained during subsequent spikes and a summation of after hyperpolarizations (Fig 9 A) and an initial adaptation (Fig 5) could occur The degree of initial adaptation and the degree of summation of after hyperpolarizations, could be varied over a wide range by changes in the constant A_{a_q} (see Kernell and Sjoholm 1972)

In maintained discharges of the present models adaptation was generally largely over after one (at weak stimuli) or a few (at stronger stimuli) initial impulse intervals (Fig 2 and 4) Within the primary range there was no further decrease of f I slope beyond the second impulse interval (Fig 3 5 and 8) In spinal motoneurones firing within the primary range most of the initial decrease in f I slope typically occurs from the first to the second impulse interval but there is usually some further decrease of f I slope beyond the second impulse interval (ratio between f I slopes for first and second interval 2 20 ± 0 16 ratio between f I slopes for second interval and steady firing 1 36 ± 0 06 mean ± SE n = 25 motoneurones) In some spinal motoneurones a gradual decrease of firing rate, associated with a decrease in f I slope might continue for as long as 1 s of steady stimulation (Kernell 1965 a) Furthermore in spinal motoneurones there may also be a late adaptation that is not associated with a change in f I slope (Granit *et al* 1963 b Kernell 1965 a) Such prolonged periods of adaptation in spinal motoneurones should be due to long time constant processes that were not included in the present model versions

The effect of maintained changes in membrane conductance (leak conductance) In spinal motoneurones maintained post synaptic conductance changes of considerable magnitude may occur without affecting the f I slope for steady firing within the primary range (Kernell 1969) Therefore it was of interest to see how the f I relation of a motoneurone like model version would be affected by a maintained conductance change

The model version of Fig 11 corresponds to that of Fig 5 except for the fact that the cellular leak conductance (g_L) was $1\,0 \times 10^{-6}$ mho for Fig 5 and $2\,0 \times 10^{-6}$ mho for Fig 11 This halving of the resting input resistance resulted as expected, in an increased threshold current for repetitive firing For the stimulus intensities of Fig 11 the slope and shape of the f I curve were however not significantly changed Interrupted lines in Fig 11 show the f I curves of Fig 5 shifted 10 nA to the right

which are capable of steady repetitive impulse firing in response to steady stimulating current (Kernell 1965 a Mishelevich 1969) *However it is important to note* that in the F H model an increase of subthreshold sodium inactivation might be marked enough to seriously disturb repetitive impulse firing without producing almost any change (less than 1 %) in the size of the initial spike elicited by a current step (Fig 1 C—D, *cf* also Fig 1 A—B in Kernell and Sjoholm 1972 and Fig 3 in Frankenhaeuser and Vallbo 1965) The observation that some spinal moto neurones with large antidromic spikes are unable of maintained repetitive firing in response to steady stimulating current (Mishelevich 1969) does not necessarily mean that such phasic properties are present in normal undamaged cells

This work has been supported by the Swedish Medical Research Council (Project No 14X 2312) Karolinska Institutets Forskningsfonder and Karolinska Institutets Datamaskintidsanslag

References

ADRIAN E D and D W BRONK The discharge of impulses in motor nerve fibres Part II The frequency of discharge in reflex and voluntary contractions *J Physiol* (Lond) 1929 *67* 119—151

AGIN D Hodkin—Huxley equations logarithmic relation between membrane current and frequency of repetitive activity *Nature* (Lond) 1964 *201* 625—626

BALDISSERA F and B GUSTAFSSON Supraspinal control of the discharge evoked by constant current in the alpha motoneurones *Brain Res* 1971 a *25* 642—644

BALDISSERA F and B GUSTAFSSON Regulation of repetitive firing in motoneurones by the afterhyperpolarization conductance *Brain Res* 1971 b *30* 431—434

BRADLEY K and G G SOMJEN Accommodation in motoneurones of the rat and the cat *J Physiol* (Lond) 1961 *156* 75—92

BROMM B Uber die repetitive Aktivitat der Schnurringmembran im Frankenhaeuser—Huxley Gleichungssystem *Pflug rs Arch ges Physiol* 1969 *307* R111

BROMM B and B FRANKENHAEUSER Repetitive discharge of the excitable membrane computed on the basis of voltage clamp data for the node of Ranvier *Pflug rs Arch ges Physiol* 1972 *332* 21—27

COOMBS J S D R CURTIS and J C ECCLES The generation of impulses in motoneurones *J Physiol* (Lond) 1957 *139* 232—249

COOMBS J S J C ECCLES and P FATT The electrical properties of the motoneurone membrane *J Physiol* (Lond) 1955 *130* 291—325

ECCLES J C Synaptic and neuro-muscular transmission *Ergebn Physiol* 1936 *38* 339—444

ECCLES J C *The Neurophysiological Basis of Mind The Principles of Neurophysiology* Clarendon Press Oxford 1953

ECCLES J C R M ECCLES and A LUNDBERG The action potentials of the alpha motoneurones supplying fast and slow muscles *J Physiol* (Lond) 1958 *142* 275—291

FRANKENHAEUSER B Computed action potential in nerve from *Xenopus laevis J Physiol* (Lond) 1965 *180* 780—787

FRANKENHAEUSER B and A F HUXLEY The action potential in the myelinated nerve fibre of *Xenopus laevis* as computed on the basis of voltage clamp data *J Physiol* (Lond) 1964 *171* 302—315

FRANKENHAEUSER B and L E MOORE The effect of temperature on the sodium and potassium permeability changes in myelinated nerve fibres of *Xenopus laevis J Physiol* (Lond) 1963 *169* 431—437

FRANKENHAEUSER B and A B VALLBO Accommodation in myelinated nerve fibres of *Xenopus laevis* as computed on the basis of voltage clamp data *Acta phys l scand* 1965 *63* 1—20

FUORTES M G F FRANK and M C BECKER Steps in the production of motoneuron spikes *J gen Physiol* 1957 *40* 735—752

GRANIT R D KERNELL and Y LAMARRE Synaptic stimulation superimposed on motoneurones firing in the secondary range to injected current *J Physiol* (Lond) 1966 *187* 40

GRANIT R D KERNELL and G K SHORTESS Quantitative aspects of repetitive firing of mammalian motoneurones caused by injected currents *J Physiol* (Lond) 1963 a *168* 911—931
GRANIT R D KERNELL and G K SHORTESS The behaviour of mammalian motoneurones during long lasting orthodromic antidromic and trans membrane stimulation *J Physiol* (Lond) 1963 b *169* 743—754
ITO M and T OSHIMA Temporal summation of after hyperpolarization following a motoneurone spike *Nature* (Lond) 1962 *195* 910—911
KERNELL D The adaptation and the relation between discharge frequency and current strength of cat lumbosacral motoneurones stimulated by long lasting injected currents *Acta physiol scand* 1965 a 65 65—73
KERNELL D High frequency repetitive firing of cat lumbosacral motoneurones stimulated by long lasting injected currents *Acta physiol scand* 1965 b *65* 74—86
KERNELL D The limits of firing frequency in cat lumbosacral motoneurones possessing different time course of afterhyperpolarization *Acta physiol scand* 1965 c 65 87—100
KERNELL D The repetitive impulse discharge of a simple neurone model compared to that of spinal motoneurones *Brain Res* 1968 *11* 685—687
KERNELL D Synaptic conductance changes and the repetitive impulse discharge of spinal motoneurones *Brain Res* 1969 *15* 291—294
KERNELL D Cell properties of importance for the transfer of signals in nervous pathways *In Excitatory Synaptic Mechanisms* Editors P ANDERSEN and J K S JANSEN Universitetsforlaget Oslo 1970 pp 269—273
KERNELL D Effects of synapses of dendrites and soma on the repetitive impulse firing of a compartmental neuron model *Brain Res* 1971 *35* 551—555
KERNELL D and H SJOHOLM Repetitive impulse firing properties of a neurone model based on voltage clamp equations compared to the properties of spinal motoneurones *Acta physiol scand* 1971 a *82* 37A—38A
KERNELL D and H SJOHOLM Initial adaptation of firing rate in a neurone model based on voltage clamp equations *Proc int Union Physiol Sci* 1971 b *9* 300
KERNELL D and H SJOHOLM Motoneurone models based on voltage clamp equations for peripheral nerve *Acta physiol scand* 1972 *86* 546—562
MISHELEVICH D J Repetitive firing to current in cat motoneurons as a function of muscle unit twitch type *Exp Neurol* 1969 *25* 401—409
PITTS R F The basis for repetitive activity in phrenic motoneurons *J Neurophysiol* 1943 *6* 439—454
SASAKI K and T OTANI Accommodation in motoneurons as modified by circumstantial conditions *Jap J Physiol* 1962 *12* 383—396
STEIN R B The frequency of nerve action potentials generated by applied currents *Proc Roy Soc B* 1967 *167* 64—86
WILSON V J and P R BURGESS Effects of antidromic conditioning on some motoneurons and interneurons *J Neurophysiol* 1962 *25* 636—650

Acta physiol scand 1973 87 57—62

From the Department of Histology University of Lund and Research Laboratories of AB DRACO Lund Sweden

Glyoxylic Acid Condensation A New Fluorescence Method for the Histochemical Demonstration of Biogenic Monoamines

By

Sture Axelsson, Anders Bjorklund Bengt Falck, Olle Lindvall and Leif Ake Svensson

Received 11 April 1972

Abstract

Axelsson S A Bjorklund B Falck O Lindvall and L A Svensson *Glyoxylic acid condensation a new fluorescence method for the histochemical demonstration of biogenic monoamines* Acta physiol scand 1973 *87* 57—62

A new methodological approach to the fluorescence microscopical visualization of biogenic monoamines based on condensation with gaseous glyoxylic acid is presented Glyoxylic acid treatment seems both to be more sensitive for the well known biogenic catecholamines and indolamines and to allow the detection of other biogenic amines—such as melatonin— which are not demonstrable with available techniques

Biogenic monoamines are biologically interesting substances which are functionally highly active also in tissues where they occur in minute amounts The fluorescence histochemistry represents an extremely sensitive methodological approach to qualitative and quantitative studies of cellular amine stores in neuronal as well as non neuronal tissues The methods based on condensation with gaseous formaldehyde (FA) are the most sensitive and in its classical form as devised by Falck Hillarp and coworkers (Falck 1962 Falck *et al* 1962 Corrodi and Hillarp 1963 1964) the formaldehyde fluorescence technique permits the visualization of very low amounts of primary catecholamines and 5 hydroxytryptamine *e g* in their intraneuronal storage sites Further developments of this method the combined formaldehyde ozone reaction (Bjorklund Falck and Hakanson 1968 Bjorklund and Falck 1969) and the acid catalyzed formaldehyde reaction (Bjorklund and Stenevi 1970 Bjorklund Nobin and Stenevi 1971) have made possible highly sensitive histochemical demonstration also of tryptamine and 3 methoxylated β phenylethylamines Apart from these formaldehyde methods fluorescence histochemical techniques have been presented for the demonstration of adrenaline—by oxidation with gaseous iodine to trihydroxyindole derivatives (Carlsson *et al* 1961 Angelakos and King 1967)—and

of histamine—by reaction with *o*-phthaldialdehyde (Juhlin and Shelley 1966 Ehinger and Thunberg 1967 Hakanson and Owman 1967) In their present shape both these methods have a lower sensitivity than the formaldehyde techniques and do not allow *e g* the demonstration of adrenaline and histamine in mammalian nervous tissues

Available fluorescence histochemical methods have obvious limitations and there is a strong demand for new supplementary techniques Particularly there is a need for new fluorescence histochemical methods for the study of biogenic amines which are known to occur in mammalian tissues, but cannot be visualized or cannot be visualized with sufficient sensitivity with existing methods Examples of such biologically interesting amines are melatonin tyramine octopamine and histamine (for references see Bjorklund Falck and Stenevi 1971) Also in instances where the differentiation between closely related compounds such as the catecholamines can be achieved in the Falck—Hillarp method only by more elaborate microspectrofluorometric techniques (*cf* Bjorklund Falck and Owman 1972) new methods for a more readily differentiation are most desirable

In the search for new fluorescence histochemical reagents and reaction mechanisms a more extensive evaluation of the histochemical usefulness of a number of reactive carbonyl compounds—*e g* aldehydes ketones carboxylic acids and *a* keto acids—has been carried out in our laboratory (Axelsson Bjorklund and Lindvall 1972) In this study glyoxylic acid (GA chemical formula HOOC CHO) was found to be one of the histochemically most interesting reagents This report present a preliminary investigation of the usefulness of glyoxylic acid for amine histochemistry

Material and methods

The study was carried out on authentic amines in histochemical models and on endogenous cellular amines *The histochemical models* were prepared from buffered solutions (pH 7 4) of human serum albumin (5 % w/v) which contained 2 0 × 10 3 M of the actual amine The solutions were sprayed as droplets on microscope slides The droplets were allowed to dry at room temperature and were then subjected to formaldehyde (FA) or glyoxylic acid (GA) treatment described below

For the tissue experiments pituitaries and pieces of submaxillary gland and distal ileum were taken from adult rats of the Sprague—Dawley strain The specimens were immediately frozen in a liquid propane propylene mixture cooled by liquid nitrogen and then freeze-dried embedded in paraffin *in vacuo* and sectioned as described by Bjorklund Falck and Owman (1972 The sections carried on microscope slides were gently deparaffinized in xylene and were allowed to react with either FA or GA vapour

The droplet models and the tissue sections were exposed to the vapours of either 5 g paraformaldehyde (Merck Darmstadt W Germany equilibrated in air with about 50 % relative humidity) in a 1 litre closed vessel at 80 C for 60 min or 800 mg glyoxylic acid monohydrate (Fluka AG Buchs Switzerland) in a 1 litre closed vessel at 100 C for 30 or 40 min The sections were then mounted in Entellan (Merck) and the models and sections were analysed in the fluorescence microscope equipped with BG 12 or UG 1 (Schott und Genossen Mainz W Germany) as primary lamp filter and Zeiss 47 + 50 or Zeiss 44 as secondary barrier filters Tissue sections exposed to heat alone were used as controls

The relative fluorescence yields in the model experiments were recorded in a Leitz microspectrofluorometer according to Bjorklund Falck and Owman (1972) Pure albumin droplets treated as above were used to obtain blank values The intensity values are given on equimolar basis

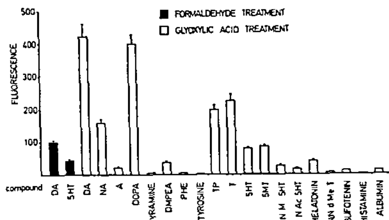
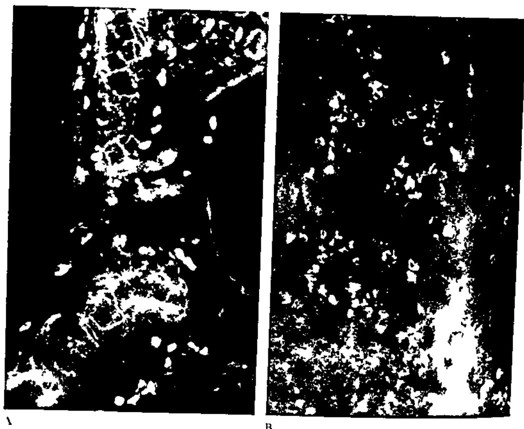


Fig 1 Relative yield of visible fluorescence obtained from some biogenic monoamines and related compounds in dried protein droplets after exposure to formaldehyde gas at +80 C for 1 h (solid bars) or glyoxylic acid gas at +100 C for 40 min (open bars) The droplets were made from buffered solutions (pH 7 4) of serum albumin (5 % w/v) containing $2\,0 \times 10^{-3}$ M of the substances Fluorescence is expressed in arbitrary units Means + S E of 2×15 droplets DA = dopamine NA = noradrenaline A = adrenaline DOPA = 3 4-dihydroxyphenylalanine DMPEA = 3 4-dimethoxyphenylethylamine PHE = phenylalanine TP = tryptophan T = tryptamine 5HT = 5 hydroxytryptamine 5 MT = 5-methoxytryptamine N Me 5HT = N methyl 5-hydroxytryptamine N Ac 5HT = N acetyl 5 hydroxytryptamine Melatonin = N acetyl 5 methoxytryptamine N N-diMe T = N N-dimethyltryptamine Bufotenin = N N dimethyl 5 hydroxytryptamine

Results and comments

In a series of preliminary experiments +100° C for 30—40 min was found to provide suitable reaction conditions for the GA treatment The standard formaldehyde treatment in the Falck—Hillarp method +80° C for 60 min was used for comparison Fig 1 gives the relative fluorescence yields from a number of biogenic amines and amino acids after treatment with GA vapour in comparison with the yields obtained from dopamine and 5 hydroxytryptamine after FA treatment

The GA treatment resulted in strong fluorescence from the primary catechol amines dopamine and noradrenaline as well as from the dimethoxylated derivative 3 4 dimethoxyphenylethylamine (DMPEA) Strong fluorescence was also obtained from primary indolamines such as tryptamine tryptophan 5 hydroxytryptamine and 5 methoxytryptamine and from the N acetylated indolamine melatonin The fluorescence induced from dopamine and 5 hydroxytryptamine by the GA treatment was considerably stronger than that obtained with the FA treatment (Fig 1) Also when comparing the present results with those given by Björklund and Stenevi (1970) on the relative fluorescence yields of various amines after FA treatment it is evident that GA treatment on the whole results in more intense fluorescence This is seen with *e g* biogenic amines such as noradrenaline tryptamine and 5 methoxytryptamine



A B

Fig 2 Tissue sections treated with GA vapour at +100 C for 40 min A Rat submaxillary gland showing a sympathetically innervated vessel surrounded by mast cells GA induced fluorescence characteristic for noradrenaline and 5 hydroxytryptamine is observed in the sympathetic nerve terminals and the mast cells respectively X 200 B GA induced fluorescence from a tryptamine like substance in cells of the rat anterior pituitary X 150

It is particularly interesting that the N acetylated amines—melatonin and N acetyl 5 hydroxytryptamine— which give no observable fluorescence upon FA treatment (Corrodi and Jonsson 1967) showed significant fluorescence after GA treatment the melatonin fluorescence being comparable to that obtained from 5 hydroxytryptamine in the FA method Another interesting finding is the relatively strong fluorescence induced by GA from the methoxylated phenylethylamine DMPEA The 3 methoxylated phenylethylamines are known to be less reactive in the first step of the fluorophore forming reaction the Pictet Spengler cyclization and no visible fluorescence is thus obtained from these compounds in the FA cyclization reaction (Corrodi and Jonsson 1967 Bjorklund and Stenevi 1970) Taken together these results indicate that GA is more efficient than FA in the fluorophore forming gas phase reactions

When the sections from freeze dried ileum submaxillary gland and pituitary were exposed to GA vapour at +100 C for 30—40 min specific GA induced fluorescence was obtained from noradrenaline containing sympathetic nerve termi

nals (Fig 2 A) from 5 hydroxytryptamine containing mast cells (Fig 2 A) and enterochromaffin cells and from a tryptamine like substance stored in cells of the adenohypophysis (Bjorklund and Falck 1969) (Fig 2 B) In preliminary microspectrofluorometric analysis these structures exhibited fluorescence excitation and emission spectra coinciding with those of the CA induced fluorophores of authentic noradrenaline (exc max /em max = 330 and 370/460 nm) 5 hydroxytryptamine (375/515—560 nm) and tryptamine (370/495—510 nm) or certain peptides with NH terminal tryptophan (380/500—535 nm) respectively In successful sections, the amine fluorescence was bright and distinct and stood out against a weakly fluorescent background and the quality of the GA fluorescence picture was quite comparable to that obtained with FA treatment according to the Falck—Hillarp method The outcome of the unstandardized treatment used in this study, however was quite variable It is evident that variations in factors such as humidity and concentration of CA in the reaction vessel the exact temperature and length of treatment *etc* will markedly influence the result of the treatment Thus similar to what is valid in the Falck—Hillarp method the GA reaction conditions must be properly standardized to give constant and reproducible results Such work is now being undertaken

In our opinion GA vapour is a most promising reagent for amine histochemistry *This reagent appears both to be more sensitive for the well known catecholamines* and indolamines by giving more intense fluorescence from these compounds and to allow sensitive detection of other biogenic amines not demonstrable with other techniques The good sensitivity of the GA treatment for melatonin is most interesting and the possibilities of devising a histofluorescence method for this N acetylated amine based on GA condensation will be the subject of further investigations

In a parallel study (Bjorklund Lindvall and Svensson 1972) it has been shown that the fluorophores formed in the reaction between CA and tryptamine are intensely fluorescent β carbolinium compounds These fluorophores are formed through an initial Pictet—Spengler cyclization reaction analogous to what takes place in the FA reaction but the fluorophore yields are markedly higher in the GA condensation reaction The high reactivity of GA compared with FA can be referred to an enhanced reactivity of the aldehyde group in combination with a very *favourable catalytic activity of the GA carboxyl* group in the reactions leading to the fluorophores

The new histochemical GA method for biogenic monoamines should be a most interesting supplement to the Falck—Hillarp method it promises to be a new and valuable tool in amine histochemistry

We thank Mrs Kerstin Fogelstrom and Mrs Birgit Haraldsson for invaluable technical assistance The study was supported by grants from the Magnus Bergvall Foundation the Alv Wiberg Foundation and the United States Public Health Service NS 06/01 06 and was carried out within a research organization sponsored by the Swedish Medical Research Council (Projects B/2 14X 712-07B and B72 14X 56 08B)

References

ANGELAKOS E T and M P KING Demonstration of nerve terminals containing adrenaline by a new histochemical technique *Nature* (Lond) 1967 *213* 391—393

AXELSSON S A BJORKLUND and O LINDVALL Fluorescence histochemistry of biogenic monoamines a study of the capacity of various carbonyl compounds to form fluorophores with biogenic monoamines in gas phase reactions *J Histochem Cytochem* 1972 *20* 435—444

BJORKLUND A B FALCK and R HÅKANSON Histochemical demonstration of tryptamine Properties of the formaldehyde induced fluorophores of tryptamine and related indole compounds in models *Acta physiol scand* 1968 Suppl 318

BJORKLUND A and B FALCK Histochemical characterization of a tryptamine like substance stored in cells of the mammalian adenohypophysis *Acta physiol scand* 1969 *77* 475—489

BJORKLUND A and U STENEVI Acid catalysis of the formaldehyde condensation reaction for sensitive histochemical demonstration of tryptamines and 3 methoxylated phenylethylamines 1 Model experiments *J Histochem Cytochem* 1970 *18* 794—802

BJORKLUND A A NOBIN and U STENEVI Acid catalysis of the formaldehyde condensation reaction for a sensitive histochemical demonstration of tryptamines and 3 methoxylated phenylethylamines 2 Characterization of amine fluorophores and application to tissues *J Histochem Cytochem* 1971 *19* 286—298

BJORKLUND A B FALCK and U STENEVI Microspectrofluorometric characterization of monoamines in the central nervous system evidence for a new neuronal monoamine like compound *Progr Brain Res* 1971 *34* 63—73

BJORKLUND A B FALCK and CH OWMAN Fluorescence microscopic and microspectrofluorometric techniques for the cellular localization and characterization of biogenic amines In *Methods in Investigative and Diagnostic Endocrinology* Ed J E Rall and I J Kopin Vol 1 The Thyroid and Biogenic Amines North Holland Publ Comp Amsterdam 1972

BJORKLUND A O LINDVALL and L Å SVENSSON Mechanisms of fluorophore formation in the histochemical glyoxylic acid method for monoamines *Histochemie* 1972 In press

CARLSSON A B FALCK N Å HILLARP G THIEME and A TORP A new histochemical method for visualization of tissue catecholamines *Med Exp* 1961 *4* 123—125

CORRODI H und N Å HILLARP Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen 1 Identifizierung der fluoreszierenden Produkte aus Modellversuchen mit 6 7 Dimethoxyisochinolinderivaten und Formaldehyd *Helv chim Acta* 1963 *46* 2425—2430

CORRODI H und N Å HILLARP Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen 2 Identifizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd *Helv chim Acta* 1964 *47* 911—918

CORRODI H and G JONSSON The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines A review on the methodology *J Histochem Cytochem* 1967 *15* 65—78

EBINGER B and R THUNBERG Induction of fluorescence in histamine containing cells *Exp Cell Res* 1967 *47* 116—122

FALCK B, Observations on the possibilities of the cellular localization of monoamines by a fluorescence method *Acta physiol scand* 1962 *56* Suppl 197

FALCK B N A HILLARP G THIEME and A TORP Fluorescence of catecholamines and related compounds condensed with formaldehyde *J Histochem Cytochem* 1962 *10* 348—354

HÅKANSON R and CH OWMAN Concomitant histochemical demonstration of histamine and catecholamines in enterochromaffin like cells of gastric mucosa *Life Sci* 1967 *6* 759—766

JULIEN L and W B SHELLEY Detection of histamine by a new fluorescent o phthalaldehyde stain *J Histochem Cytochem* 1966 *14* 525—528

Acta physiol scand 1973 87 63—68

From the Department of Physiology Karolinska Institutet Stockholm Sweden

Spectral Sensitivity of the Compound Eye in a Moth Intra- and Extracellular Recordings

By

GORAN STRUWE

Received 11 April 1972

Abstract

STRUWE G *Spectral sensitivity of the compound eye in a moth Intra and extra cellular recordings* Acta physiol scand 1973 87 63—68

The spectral sensitivity of the compound eye in a moth (*Manduca sexta*) was tested electrophysiologically by extracellular recordings in the wavelength region 310 to 650 nm a) during dark adaptation and b) during exposure to violet (408 nm) and red (680 nm) light. Sensitivity maxima were found at 370 to 390 nm 450 to 470 nm and 530 to 550 nm. During exposure to violet light a larger decrease in sensitivity was found for the maxima at 370 to 390 nm and 450 to 470 nm compared to that at 530 to 550 nm During exposure to red light a larger decrease in sensitivity was found for the latter maximum The results are interpreted as evidence for at least two photoreceptor types with different spectral sensitivity maxima Intracellular recordings from single photoreceptors showed maxima in the same wavelength regions as those found extracellularly The spectral sensitivities obtained were approximately similar The homogeneity of the intracellular recordings is suggested to be due to interreceptor coupling

The superposition compound eye of moths is anatomically different from the apposition compound eye of butterflies For instance in the moth eye the crystalline cones are separated from the rhabdoms by extensions from the photoreceptors called the crystalline tracts which are surrounded by a migrating screening pigment (Exner 1891 Yagi and Koyama 1963) The results of behavioural experiments indicate that although moths are active in dim light while butterflies are not both insect groups are capable of colour vision (Knoll 1925 Ilse 1928 Schlegdendal 1934 Schremmer 1941 Crane 1955 Ilse and Vaidya 1956 Obara 1970 Swihart 1971) Intracellular recordings show that wavelength discrimination in some insects with apposition compound eyes is effected by photoreceptors with different spectral sensitivities (for ref see p 5) No such experiments have been made on the compound eyes of moths or butterflies

In a preliminary report three photoreceptor types with different spectral response maxima were suggested for the compound eye of the moth *Manduca sexta* (Boethius *et al* 1968) The present investigation which i an extension of the previous st

concerns the spectral response of single photoreceptors in *Manduca sexta* and the spectral sensitivity of its compound eye during selective adaptation The results suggest that the eye contains at least two photoreceptor types with different spectral sensitivities

Material and methods

The experiments were made on the compound eye of the moth *Manduca sexta* (Lepidoptera, Sphingidae) The moths were dark adapted for at least 30 min before the experiment. A maximal 'glow' showed that the screening pigment had reached the extreme distal position (Hoglund 1966) The compound eye was severed from the body and fixed to a filter paper moistened with saline

Stimulation Test flashes (200 ms) from a xenon arc (Osram XBO 450) passed through double line interference filters (Schott PIL and DEPIL) The transmission maxima of the interference filters were spaced at intervals about 20 nm between 310 and 650 nm The monochromatic test flashes were adjusted to equal photon content at each wavelength by neutral density filters (Balzers) The average deviation from equal photon content was 0 07 log units (maximal deviation 0 15 log units and SEM 0 07) The light stimulus was transmitted through a light guide (Zeiss diameter 2 mm) the end of which was placed close to the eye The illumination covered 2/3 of the corneal surface Adapting lights were obtained by placing broadband interference filters (Balzers) in front of a tungsten lamp The intensity of the adapting light was adjusted by changing the voltage Test flashes were presented consecutively from 350 to 650 nm at intervals of 10 s in all recordings

Recording The moistened filter paper was connected to a reference electrode (Ag AgCl) In *intracellular* recordings a glass (Corning) microelectrode filled with 3 M KCl (impedance 30 to 50 Mohm) was advanced through a hole made in the cornea. The microelectrode was connected to a preamplifier (input resistance 10^{1} ohm) and an oscilloscope (Tektronix 502 B) In *extracellular* recordings an active electrode (Ag AgCl diameter 0 1 mm) was inserted just below the cornea and connected to a preamplifier (Grass P 6 12) The rest of the recording equipment was similar to that used for the intracellular recordings

Experimental procedure In *intracellular* recordings penetration of a single cell was indicated by a drop in D C potential of about 50 mV and a depolarizing response to light stimulation The spectral efficiency (amplitude of photoreceptor potential *vs* wavelength of stimulating light) was measured after the membrane potential had been approximately constant for 3 min. In extracellular recordings the spectral efficiency of the compound eye was recorded consecutively as follows (1) during maximal dark adaptation, (2) during exposure to a monochromatic adapting light (wavelength 680 nm bandwidth 20 nm) (3) after 10 min of dark adaptation (4) during exposure to a monochromatic light (wavelength 408 nm bandwidth 10 nm) and (5) again after 10 min dark adaptation The relation between stimulus intensity (test flashes 200 msec of white light) and response amplitude was determined during each experimental stage (1—5) Spectral sensitivity was calculated from the spectral efficiency and stimulus response relationship as described by Autrum and von Zwehl 1964

Results

In 7 expts the spectral efficiency and stimulus response relationship was recorded extracellularly from the dark adapted compound eye and during exposure to monochromatic adapting lights in the wavelength regions 680 nm and 408 nm (see Methods) The spectral sensitivities were calculated for a threshold response of 50 μV

The result of a typical experiment is illustrated in Fig 1 The figure shows the spectral sensitivity of the dark adapted eye before exposure to the adapting lights (continuous line) Spectral sensitivity maxima are seen at 370 to 390 nm, 450 to 470 nm and 530 to 570 nm During adaptation with monochromatic light of about 680 nm sensitivity maxima were observed in the same wavelength regions (broken line) although the sensitivity decreased at all tested wavelengths The sensitivity

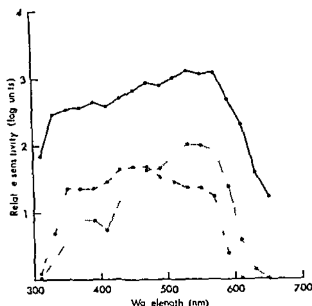


Fig 1 Spectral sensitivity of the dark adapted eye (continuous line) and during exposure to violet (dotted line) or red light (broken line) 1 exp

decrease was larger for the maximum at 530 to 570 nm as compared to that of other maxima During adaptation with monochromatic light about 408 nm a decrease in sensitivity is again observed at all tested wavelengths (dotted line) In this case the decrease in sensitivity is larger for the maxima at 370 to 390 nm and 450 to 470 nm compared to the maximum at 530 to 570 nm After exposure to the adapting lights the eye was dark adapted for 10 min and the spectral sensitivity was found to be approximately equal to that measured before exposure to the adapting light (continuous line)

When the eye was exposed to an adapting light of low intensity compared to that used in the experiment described above the decrease in sensitivity did not affect all the spectral sensitivity maxima On the other hand if the intensity of the adapting light was increased an almost equal decrease in sensitivity was found at all sensitivity maxima

For reasons discussed below the result obtained in the experiment illustrated in Fig 1 suggests that the compound eye contains at least two types of photoreceptors with different spectral sensitivity maxima Intracellular recordings were made to determine the spectral sensitivity of these photoreceptors The spectral response was recorded intracellularly from 28 photoreceptor cells In all recordings the impaled photoreceptor responded to all wavelengths tested (310 to 650 nm) and sensitivity maxima were observed in the wavelength regions 370 to 390 nm 450 to 470 nm and 530 to 550 nm. The relative amplitude of the different maxima was found to differ somewhat between individual experiments Fig 2 shows the mean spectral response (expressed as per cent of maximal amplitude) of all recordings

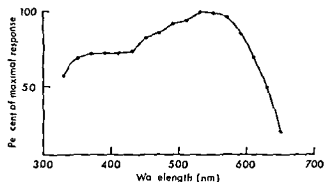


Fig 2 Spectral response as recorded intracellularly from single photoreceptor cells in the compound eye Mean of 5 expts expressed in per cent of maximal response at 530 nm

in which a depolarization of more than 3 mV was observed Maxima were found at 370 to 390 nm and at 530 to 550 nm Considering the spectral sensitivity as recorded by gross electrodes (Fig 1 continuous line) the slight discontinuity at 450 to 470 nm in Fig 2 may represent a third maximum

Discussion

In extracellular recordings on the dark adapted compound eye (Fig 1) sensitivity maxima were found at 370 to 380 nm 450 to 470 nm and at 530 to 570 nm During exposure of the eye to monochromatic adapting lights (about 680 and 408 nm) the sensitivity at some maxima decreased more than others although the sensitivity decreased to all wavelengths tested

Before interpreting the present results as possible evidence for the existence of more than one type of photoreceptor it should be considered if the variations in spectral sensitivity observed during illumination were due to factors other than photoreceptor adaptation Inward pigment movement during light adaptation causes a decrease in sensitivity of the eye (Bernhard and Ottoson 1960) Such movements by the screening pigment were small in the present experiments since after a completed experiment the sensitivity was found to be approximately equal to that of the dark adapted eye In addition inward pigment movement in the compound eye of moths causes only small variations in spectral sensitivity within the wavelength region tested here (Hoglund and Struwe 1970)

The selective changes in the spectral sensitivity during exposure to the adapting lights used therefore show that the maxima found for the compound eye are caused by at least two types of photoreceptors with different spectral sensitivity This interpretation agrees with the result of microspectrophotometric measurements on the compound eye of the moth *Deilephila elpenor* (Hamdorf Hoglund and Langer 1971) The microspectrophotometric measurements indicate the existence of three different photopigments with maximal absorption in the wavelength regions 370 to 390 nm, 450 to 470 nm and 530 to 550 nm

At the intensity of the adapting light used in the experiment illustrated (Fig 1)

or at higher intensities a decrease in sensitivity was observed at all wavelengths tested The effect may be due to the absorption of the adapting light by several photopigments If so a decrease in sensitivity at all wavelengths would also occur at low intensities of the adapting light *This was, however not the case.*

Alternatively, the decrease in sensitivity at all wavelengths may be explained by an electrical coupling between the photoreceptor cells in one ommatidium Such interreceptor coupling causes dissipation to the impaled cell of current generated in adjacent cells The individual function of a single photoreceptor is thereby impeded Electrical coupling has been demonstrated in the compound eyes of the honeybee *Apis* and the grasshopper *Locusta* (Shaw 1969) *Shaw suggested that the inter* receptor coupling takes place between the rhabdomeric tubules The photoreceptors in the moth eye have well developed rhabdomeric villi and the outer lamellae of the plasmalemma of adjacent cells are often fused (Gemne personal communication) Interreceptor coupling may therefore be large in the moth eye If the coupling increases during illumination the sensitivity to all wavelengths would also decrease *during selective adaptation as found in the present study*

Intracellular recordings from single photoreceptors were made in order to identify the spectral response of the photoreceptor classes indicated by the result of extra cellular experiments Recordings with spectral response maxima at about 370 to 390 nm 450 to 470 nm and 530 to 550 nm were found The relation between the amplitude of these maxima varied somewhat in individual recordings but the spectral response was approximately similar in all In intracellular recordings from the compound eye of other insects photoreceptors with different spectral sensitivity maxima have been found (Autrum and von Zwehl 1964 Burkhardt 1962 Bennet Tunstall and Horridge 1967 Bruckmoser 1968 Autrum and Kolb 1968 Horridge 1969 Goldsmith and Mote 1970 Eguchi 1971) The difference between the present results and those obtained on other species may be explained in different ways For instance damage to the impaled cell by the microelectrode may have caused a current leakage from adjacent cells The spectral response of adjacent cells may then affect that of the impaled cell (Autrum and von Zwehl 1964) Alternatively the spectral response of adjacent cells may be electrically conducted to the impaled cell by interreceptor coupling as demonstrated in other insect species (see also above) (Shaw 1969)

If the interreceptor coupling decreases at low intensities of the stimulating light the photoreceptor cells will become electrically isolated Such a decrease in electrical coupling may explain that some recordings with only one spectral response maximum were found in this moth (Boethius *et al* 1968) In these experiments lower stimulating light intensities than tho e in the present study were used

The results of behavioural experiments indicates that a related moth *Deilephila livornica* is capable of colour vision (Knoll 1925) Such wavelength discrimination requires at least two independent photoreceptors with different spectral sensitivity If the interreceptor coupling is low in dim light the moth may be capable of colour vision during its activity at dusk

This work was supported by the Swedish Medical Research Council grant No B71 14X 104 06B and 07C Sallskapet for Medicinsk Forskning and Reservationsanslaget

References

AUTRUM H and V v ZWEHL Die spektrale Empfindlichkeit der einzelnen Sehzellen des Bienenauges *Z vergl Physiol* 1964 *48* 357—384

AUTRUM H and G KOLB, Die spektrale Empfindlichkeit einzelner Sehzellen der Aeschniden. *Z vergl Physiol* 1968 *60* 450—477

BENNET R R J TUNSTALL and G A HORRIDGE Spectral sensitivity of single retinula cells of the locust *Z vergl Physiol* 1967 *55* 195—206

BERNHARD C G and D OTTOSON Comparative studies on dark adaptation in the compound eyes of nocturnal and diurnal Lepidoptera. *J gen Physiol* 1960 *44* 195—203

POLTHIUS J S D CARLSON G HOGLUND and G STRUWE Spectral efficiency of single photoreceptor cells of the moth (*Manduca sexta*) *Acta physiol scand* 1968 *74* C 30

BRUCKMOSER P Die spektrale Empfindlichkeit einzelner Sehzellen des Ruckenschwimmers *Notonecta glauca* L (Heteroptera) *Z vergl Physiol* 1968 *59* 187—204

BURKHARDT D Spectral sensitivity and other response characteristics of single visual cells in the arthropod eye *Symp Soc exp Biol* 1962 *16* 86—109

CRANE J Imaginal behaviour of a Trinidad butterfly *Heliconius erato hydara* Hewitson, with special reference to the social use of color *Zoologica* 1955 *40* 167—196

EGUCHI E Fine structure and spectral sensitivities of retinular cells in the dorsal sector of compound eyes in the dragonfly *Aeschna Z vergl Physiol* 1971 *71* 201—218

EXNER S *Die Physiologie der facettirten Augen von Krebsen und Insecten* Deuticke Leipzig and Vienna 1891

HAMDORF K G HOGLUND and H LANGER Mikrophotometrische Untersuchungen an der Retinula des Nachtschmetterlings *Deilephila elpenor Verh dtsch Zool Ges* 1971 In press

HORRIDGE G A Unit studies on the retina of dragonflies *Z vergl Physiol* 1969 *62* 1—37

HOGLUND G Pigment migration light screening and receptor sensitivity in the compound eye of nocturnal *Lepidoptera Acta physiol scand* 1966 *69* Suppl 282

HOGLUND G and G STRUWE Pigment migration and spectral sensitivity in the compound eye of moths *Z vergl Physiol* 1970 *67* 229—237

ILSE D Uber den Farbensinn der Tagfalter *Z vergl Physiol* 1928 *8* 658—692

ILSE D and V VAIDYA Spontaneous feeding response to colours in *Papilio demoleus* L. *Proc Indian Acad Sci* 1956 *43 B* 23—31

KNOLL F Lichtsinn und Blutenbesuch des Falters von *Deilephila livornica Z vergl Physiol* 1925 *2* 329—380

MOTE M I and T H GOLDSMITH Spectral sensitivities of color receptors in the compound eye of the cockroach *Periplaneta J exp Zool* 1970 *173* 137—146

OBARA Y Studies on the mating behaviour of the white cabbage butterfly *Pieris rapae crucivora* Boisduval III Near ultra violet reflection as the signal of intraspecific communication *Z vergl Physiol* 1970 *69* 99—116

SCHLEGTENDAL A Beitrag zum Farbensinn der Arthropoden *Z vergl Physiol* 1934 *20* 545—581

SCHREMMER F Sinnesphysiologie und Blumenbesuch des Falters von *Plusia gamma* L *Zool Jahr Abt Syst* 1941 *74* 375—434

SHAW S R Interreceptor coupling in ommatidia of drone honeybee and locust compound eyes *Vision Res* 1969 *9* 999—1029

SWIHART C A Colour discrimination by the butterfly *Heliconius charitonius* LINN *Anim Behav* 1971 *19* 156—164

YAGI N and N KOYAMA *The compound eye of Lepidoptera* Shinkyo Press Tokyo 1963

Acta physiol scand 1973 87 69—77

From the Brain Research Laboratory E Blocket the University Hospital of Lund and from the Department of Surgery the University of Lund Sweden

Cerebral Blood Flow in Ischemia Caused by Carotid Artery Ligation in the Rat

By

Bo Eklof and Bo K. Siesjo

Received 12 April 1972

Abstract

Eklof B and B K Siesjo *Cerebral blood flow in ischemia caused by carotid artery ligation in the rat* Acta physiol scand 1973 *87* 69—77

In order to study if bilateral ligation of the carotid arteries either alone or combined with moderate arterial hypotension is accompanied by a macroscopically uneven brain perfusion, the regional cerebral blood flow (rCBF) was measured using the antipyrine ^{14}C method with direct counting of the radioactivity in the tissue. Ligation of the carotid arteries at normal blood pressure decreased blood flow in the frontal parietal regions by about 50 % in both hemispheres At a mean blood pressure of 100 mm Hg the ligation caused a further reduction in flow but the results were variable and gross differences often occurred between the hemispheres At 70 mm Hg in blood pressure carotid occlusion drastically reduced the frontal parietal rCBF but flow in the occipital and basal temporal regions was usually better upheld. It is concluded that in the rat combined carotid artery ligation and moderate hypotension gives rise to a macroscopically inhomogenous reduction in rCBF and that regions of nonperfusion may coexist with adequately perfused areas

In a previous communication (Eklof and Siesjo 1972 a) we reported that bilateral carotid artery ligation in the rat reduced the cerebral blood flow as calculated from the a v differences for O_2 and CO_2 to about 50 per cent of the normal The decrease in flow was not associated with significant changes in the energy state of the tissue When the ligation of the carotid arteries was performed after a reduction of the mean arterial blood pressure to 100 and 70 mm Hg respectively a marked deterioration of the energy state occurred in spite of the fact that the cerebral venous P_{O_2} remained at 25—30 mm Hg The dissociation between the energy state and the venous P_{O_2} as well as between the intracellular pH values derived from the CO_2 method and those predicted from the tissue lactate values (Eklof and Siesjo 1972 b) suggested an inhomogenous reduction in cerebral blood flow Thus the results obtained indicate that the ischemic model used may cause a circulatory pattern with cessation of flow in some parts of the tissue and with preserved perfusion in others However the results did not allow conclusions as to whether the inhomogeneity involved the microcirculation or relatively large tissue regions

In the present report the regional cerebral blood flow (rCBF) was measured with the objective of studying possible inhomogeneities of flow at the macroscopic level using the experimental model described in the two preceding communications The main objective of the study was to evaluate the presence of differences in flow between the two hemispheres The main comparison was made between brain regions supplied by the middle cerebral artery, but control measurements were made on areas supplied by the posterior cerebral artery The rCBF was measured with a modification of the antipyrine ^{14}C method (Reivich *et al* 1969) utilizing direct counting of the ^{14}C activity in dissected brain regions The rCBF values obtained will be correlated to some of the findings obtained in the previous reports (Eklof and Siesjo 1972 a and b)

Methods

A *Operative and sampling techniques* The experimental techniques were similar to those described in the preceding reports In general male wistar rats were anesthetized with diethyl ether and maintained on 70 % N_2O and 30 % oxygen during artificial ventilation The body temperature was kept close to 37 C Both femoral arteries and femoral veins were cannulated with PE 50 catheters One of the arterial catheters was used for continuous recording of the blood pressure and for anaerobic sampling of blood The other catheter was used for bleeding the animals into a constant pressure reservoir and for sampling of blood for measurements of the activity of antipyrine ^{14}C Before those samples were taken the catheter was cut to a length of 15—20 mm One of the femoral veins was used for infusion of the antipyrine ^{14}C Before those samples were taken the catheter was cut to a length of 15—20mm One of the femoral veins was used for infusion of the antipyrine ^{14}C (see below) and the other for injection of saturated potassium chloride at the end of the experiment

In order to allow carotid artery ligation the common carotid arteries were dissected free in the neck and ligatures were placed around the vessels for subsequent occlusion

B *Experimental protocol* After the operative procedures the animals were allowed a 20—30 min steady state period During that period at least 2 arterial samples were taken for analyses of P_{CO_2} pH and P_{O_2} The respirator was adjusted to give arterial P_{CO_2}s of 35—40 mm Hg and it was controlled that all animals had a Pa_{O_2} of more than 100 mm Hg

The animals were then divided into 4 groups In one group no carotid artery ligation was performed and the animals were left for another 30 min In the second group the carotid arteries were occluded and the blood pressure was allowed to attain its spontaneous value. In group 3 and 4 the animals were bled to mean arterial blood pressures of 100 and 70 mm Hg respectively before the carotid arteries were ligated In all animals blood was sampled for analyses of P_{CO_2} P_{O_2} and pH 5 and 30 min respectively after the ligation and samples were taken at comparable times in the nonligated animals Thirty minutes after the ligation about 1 ml of an isotonic NaCl solution containing antipyrine ^{14}C (administered activity 50 μCi/kg b w) was infused i v during 60 s Arterial blood was sampled in glass capillaries every 5 s during the 60 s infusion period Immediately after the last sample had been taken (at 61 s) 1 ml of saturated KCl was rapidly injected i v and the rat was decapitated within 2—3 s. The heads were then frozen in liquid nitrogen and the brains were chiselled out in the frozen state and stored at −80 C until analysed If the arterial blood pressure fell by more than 10 mm Hg during the sampling period the rats were excluded

C *Analytical techniques* P_{O_2} P_{CO_2} pH and hemoglobin concentration were measured as described previously (Eklof and Siesjo 1972 a

For counting of the ^{14}C activity in the blood samples the glass capillaries which contained 25—50 μl blood were emptied into preweighed scintillation vials containing Soluene® and isopropanol The vials were immediately reweighed to determine the amount of blood Hydrogen peroxide was added to decrease colour quenching and Instagel® to provide a scintillation system The vials were then left in the dark and counted with the tissue samples

The brain samples were dissected and prepared for analyses in a box maintained at −15 C, which had an inspection window and a torsion balance In all instances a frontal parietal part of one hemisphere containing both grey and underlying white matter was cut out divided into 2 equal pieces and weighed Each piece (hereafter called the chunk) weighed about 100 mg

TABLE I Mean rCBF in lightly anesthetized rats Comparison between the 2 hemispheres in 6 animals

	rCBF ml/100 g/min		
	Ipsilateral hemisphere (n=6) Chunk I	Chunk II	Contralateral hemisphere (calculated from aliquots of the ground hemisphere) (n=15)
Mean	61 0	64 2	62 2
SE	1 4	1 9	1 3

and the 2 pieces together represent the same brain region previously used in the laboratory for metabolite analyses (Eklof MacMillan and Siesjo 1972 a and b)

The antipyrine ^{14}C method has previously not been applied to direct counting of brain tissue. In order to study how representative the tissue chunk is for the mean flow of the hemisphere a series of unligated control rats were used to compare the ^{14}C activity of the chunk and the activity of the contralateral hemisphere which was ground to a powder In all ligated animals however identical chunks from the 2 hemispheres were compared In addition to the frontal parietal chunks which mainly represent tissue supplied by the middle cerebral artery about 40—60 mg pieces from the occipital and temporo-basal regions mainly supplied from the posterior cerebral artery were also dissected and analysed in some of the animals

Each piece of brain tissue or an aliquot of the ground hemisphere were weighed and put into plastic vials containing Soluene® and allowed to stand in the dark for 24 h when the tissue was dissolved Instagel® was then added and the ^{14}C activity of the brain and blood samples were counted in a Nuclear Chicago scintillation counter Appropriate corrections were made for quenching using external standardization.

Calculations

The rCBF was calculated according to the formula of Kety (1960)

$$C_i(T) = \lambda \ K_i \int_O^T C_a \ e^{-k_i(T-t)} dt$$

here C (T) is the concentration of antipyrine ^{14}C in the brain tissue at time T which in these studies was 61 s λ is the blood brain partition coefficient for antipyrine ^{14}C with a value of 0 99 (Reivich *et al* 1969) K is the rate of blood flow per unit weight of brain tissue multiplied by the reciprocal of the partition coefficient for that tissue and C is the concentration of antipyrine ^{14}C in arterial blood Since the arterial catheter used was very short (15—20 mm) no correction was made for the smearing factor of the sampling catheter A program was written for the Varian computor 620/i to calculate by means of the trapezoid rule the value of C (T) for various values of K_i chosen according to a simple algorithm to reach the proper K

Results

In order to correlate the calculated blood flow in the frontal parietal chunk with the mean blood flow in the hemisphere 6 unligated animals with a Pa_{CO_2} of 35—40 mm Hg a Pa_{O_2} of > 100 mm Hg and a mean arterial blood pressure of > 120 mm Hg were analysed as described above (see Methods) Table I shows (1) that the rCBF as calculated from aliquots of the ground hemisphere was very constant (2) that there were very small differences between the 2 pieces of the frontal-parietal chunk and (3) that the mean rCBF calculated from the ^{14}C activity in the chunk was identical to the mean CBF of the contralateral hemisphere

TABLE II rCBF in 17 lightly anaesthetized rats Mean rCBF calculated from pieces of frontal parietal chunks as well as from aliquots of ground brain tissue In addition to P_{O_2}, P_{CO_2} and pH the table also gives the body temperature the arterial hemoglobin concentration (Hb) and the mean arterial blood pressure (MABP) Means ± S E Figures within parenthesis indicate number of experiments

T	Hb	Pa_{CO_2}	Pa_{O_2}	pH	MABP	Mean rCBF	Occipital cortical rCBF
C	g %	mm Hg	mm Hg		mm Hg	ml/100g min	ml/100g min
37 0 ±0 2	15 2 ±0 1	37 7 ±0 3	128 ±3 5	7 40 ±0 004	159 ±2 6	64 6 ±0 8 (45)	88 7 ±2 2 (13)

TABLE III Mean rCBF in rats with bilateral carotid artery ligation for 30 min calculated from pieces of frontal parietal chunks from both hemispheres Basic physiological data are listed to the left as means ± S E

	rCBF ml/100g min			
	Left hemisphere		Right hemisphere	
	Chunk I	Chunk II	Chunk I	Chunk II
T 37 0 C ±0 2	32	32	33	35
MABP 1/0 mm Hg ±6	31	31	30	32
Hb 15 1 g ±0 1	41	39	42	37
Pa_{CO_2} 36 3 mm Hg ±1 4	17	13	23	?6
Pa_{O_2} 117 mm Hg ±5	29	30		
pH 7 33 ±0 02	37	38	40	
	50	54		
	32	29	44	34
	35	35		
	33	31	24	24
Mean	33 4		32 6	
S E M	±2 0		±1 9	

In all 17 unligated animals with a normal acid base state were analysed usin either the chunk method or aliquots of ground hemisphere (Table II) For the e animals a mean rCBF of 64 6 ml/100 g min was obtained In some animals an occipital region was dissected so as to give predominantly cortical tissue and in this tissue a higher rCBF was obtained (88 7 ± 2 2 ml/100 g min) Thus although the frontal parietal chunk probably contained more cortical grey matter than subcortical white substance the comparison with the occipital cortical flow indicates that the chunk method gives a mean flow for grey and white matter

Table III shows the flow values calculated for the ligated animals in which the blood pressure was allowed to attain its spontaneous value The carotid artery ligation decreased the rCBF of a hemisphere to between 23 and 81 % of the normal However in most of the animals the rCBF was decreased to about 50 % of the

TABLE IV rCBF in rats bled to a mean arterial blood pressure of 100 mm Hg with bilateral carotid artery ligation for 30 min calculated from pieces of frontal parietal chunks from both hemispheres In 2 animals the rCBF was calculated for occipital and temporo-basal areas as well

	rCBF ml/100g min							
	Left hemisphere				Right hemisphere			
	Chunk I	Chunk II	Occipital	Temporo-basal	Chunk I	Chunk II	Occipital	Temporo-basal
T 36 7 C ±0 2	33	37			8	5		
	40	37			6	5		
Hb 13 9 g ±0 5	1	2			46	42		
	14	16			5	3		
Paco 33 1 mm Hg ±0 9	42	53			70	68		
	5	7			40	40		
Pao 122 mm Hg ±5	8	4			1	3		
	14	12			10	8		
pH 7 24 ±0 01	31	29	34	33	7	8	20	24
	46	47	59	47	39	44	45	49

TABLE V rCBF in rats bled to a mean arterial blood pressure of 70 mm Hg with bilateral carotid artery ligation for 30 min calculated from pieces of frontal parietal chunks from both hemispheres In 6 animals the rCBF was calculated for occipital and temporo basal areas as well

	rCBF ml/100g min							
	Left hemisphere				Right hemisphere			
	Chunk I	Chunk II	Occipital	Temporo basal	Chunk I	Chunk II	Occipital	Temporo-basal
T 36 8 C ±0 2	8	5			29	28		
Hb 13 1 g ±0 4	2	6			1	1		
Paco 30 7 mm Hg ±1 4	5	1		25	1	0		
	3	4			10	8		
	7	4			40	25		
	2	2			8	3		9
Pao 131 mm Hg ±4	1	2	10	21	1	1	0	4
	7	3	18	19	3	12		45
pH 7 19 ±0 03	2	1	14	35	0	1		5
	5	8	17	23	9	22	[illegible]	73

normal and the results are thus in very good agreement with the CBF values derived from the a v O and CO differences in the preceding communication (Eklof and Siesjo 1972 a) A *comparison of the rCBF values calculated for the 2 hemispheres* shows that the decrease in flow was about the same on the 2 sides However in one out of the ten animals the rCBF was markedly reduced and in this animal there was a relatively large difference in rCBF between the two hemispheres

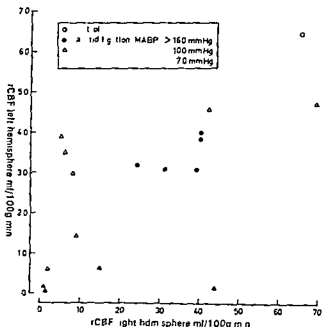


Fig 1 A comparison between rCBF in the frontal parietal area in each brain calculated from the mean rCBF value of the 2 frontal parietal chunks in the left and right hemisphere respectively

In the ligated group maintained at a mean arterial blood pressure of 100 mm Hg (Table IV) there was a very variable decrease in rCBF and in this group appreciable side differences appeared Thus the calculated rCBF values varied between close to zero and normal values and a very low flow on one side was often associated with a relatively high rCBF value on the other side Since there were very small difference between the 2 pieces of the chunks the inhomogeneity of flow seemed to affect the chunk as a whole However the results obtained on at least one of the rats suggested that there were differences in flow within the hemisphere The variability in flow at this blood pressure level agrees well with the corresponding variability in metabolic state as reported previously (Eklof and Siesjo 1972 a and b)

At a mean blood pressure of 70 mm Hg (Table V) almost all animals showed a very pronounced fall in rCBF and the results therefore corroborate the previous findings of an almost invariable derangement of the energy state at this pressure (Eklof and Siesjo 1972 a and b) In this group there were sometimes differences in rCBF between the 2 pieces of a chunk suggesting an inhomogenous fall in flow with in the chunk In addition analyses on occipital and temporobasal regions indicated that these regions had a better preserved circulation than those supplied by the middle cerebral artery In the preceding communications (Eklof and Siesjo 1972 a and b) it was concluded that the ischemic model used gave rise to a regional cessation of flow The present results are completely in agreement with this conclusion since many of the rCBF values calculated were close to zero (see also Table IV)

In Fig 1 the rCBF values calculated for the chunks from the right and the left hemisphere have been compared The figure emphasizes the symmetrical decrease

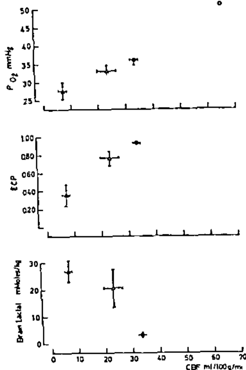


Fig 2 A correlation between the calculated gross means of rCBF for the four groups (Explanation of symbols see Fig 1) and cerebral venous oxygen tension energy charge potential of the adenine nucleotide pool and concentration of brain lactate Means ± S E

in flow in the ligated group maintained at a normal blood pressure as well as the pronounced asymmetry in the hypotensive groups There was no preponderence for any of the hemispheres In all 3 groups a gross mean for rCBF could be calculated by using the values for the 2 hemispheres Although this gross mean may have little physiological meaning it can be compared with the corresponding metabolic data to provide a very rough relationship between CBF and metabolic state Fig 2 shows a comparison between on one hand the mean rCBF and on the other the cerebral venous P_{O_2} the energy charge potential and the lactate content of the brain as reported in the previous communications (Eklof and Siesjo 1972 a and b) The figures indicate that the energy state of the tissue markedly deteriorates when the rCBF is reduced to 30—40 % of normal It should be remarked that the same rough relation hip holds if rCBF is derived from only one of the hemispheres

Discussion

As remarked in the introduction the present experiments had the objective of elucidating if carotid ligation and induced hypotension in the rat gives rise to a grossly inhomogenous reduction in cerebral blood flow Before evaluating the main results it seems appropriate to discuss the rCBF method used

In ischemic situations which may be characterized by an inhomogenous reduction in CBF, and especially if totally unperfused areas exist, it seems preferable to use a CBF method which measures the accumulation rather than the clearance of a suitable isotope marker This is due to the fact that since an unperfused region receives no isotope it will not contribute to the clearance of the isotope The antipyrine ^{14}C mehod (Reivich *et al* 1969) which is based on the principles laid down by Kety (1951 see also Landau *et al* 1955 Freygang and Sokoloff 1958) seem well suited for ischemic situations since it allows autoradiographic evaluation of the antipyrine ^{14}C activity in the tissue This has made it possible to use the method for studies of absolute rCBF values in many brain regions and to show that the rCBF even within the grey matter varies appreciably between different regions (Reivich *et al* 1969 Kennedy 1972)

Due to the small size of the rat brain it is relatively difficult to resolve regional differences in rCBF with the autoradiographic technique We therefore chose direct scintillation counting of the ^{14}C activity in the tissue As long as areas with a uniform flow are not dissected this procedure has the disadvantage that it yields a mean flow of questionable physiological significance but it has the advantage of providing rapid and accurate values for the ^{14}C activity in the tissue Furthermore the procedure allows dissection in the cold of identical brain regions for rCBF and for metabolite analyses

The present results have shown that bilateral carotid artery ligation in the rat reduces rCBF in a frontal parietal piece mainly supplied by the middle cerebral artery to about 50 % of normal and further that the decrease in flow affects both hemispheres about equally When the ligation was performed after a previous reduction in blood pressure to 100 or 70 mm Hg there was a further decrease in flow but this often affected the sides unequally In the blood pressure 100 group which showed a pronounced variability in response the inhomogeneities were sometimes such that one hemisphere could show an almost normal flow while the other was almost totally ischemic In the blood pressure 70 group analyses of occipital and basal temporal regions showed inhomogeneities within the hemisphere and such inhomogeneities were also seen within the frontal parietal region analysed Therefore we must conclude that the cerebral ischemia induced by combined bilateral carotid artery ligation and arterial hypotension involves a grossly inhomogeneous decrease in flow affecting hemispheres or large parts of the hemispheres This does not exclude the presence of microinhomogeneities as well but it emphasizes the necessity to analyse regional cerebral blood flow in the brain in various forms of ischemia using animal species with a vascular supply which is more similar to that of man

The concept of an inhomogenous decrease in flow in ischemia is not a new one since there are a number of morphological studies showing a preferential effect on the border zone areas between the distribution territories of the major cerebral arteries (Lindenberg 1963 Brierley *et al* 1969) However since large differences in rCBF were often found between identical regions in the 2 hemispheres the

present results do not indicate that the decrease in flow in the present ischemic model preferentially affected these "water shed" areas It will remain for future studies to delineate the exact distribution of the ischemic lesions and to analyse the pathophysiological factors which contribute to cause them

This study was supported by grants from the Swedish Medical Research Council (Project No B72 14X 2179 04 and B7 14X 263-08B) from the Swedish Bank Tercentenary Fund by U.S PHS Grant No RO1 NS 07838-03 from NIH and by Tore Nilson s Fund

References

BRIERLEY J B A. W BROWN B J EXCELL and B S MELDRUM Brain damage in the rhesus monkey resulting from profound arterial hypotension I Its nature distribution and general physiological correlates *Brain Res* 1969 *13* 68—100

EKLOF B and B K. SIESJO The effect of bilateral carotid artery ligation upon the blood flo and the energy state of the rat brain *Acta physiol scand* 1972 a. *86* 155—165

EKLOF B and B K SIESJO The effect of bilateral carotid artery ligation upon acid base parameters and substrate levels in the rat brain *Acta physiol scand* 1972 b *86* 5 8—538

EKLOF B V MACMILLAN and B K. SIESJO Cerebral energy state and cerebral venous P_{O_2} in experimental hypotension caused by bleeding *Acta physiol scand* 1972 a. *86* 515—527

EKLOF B V MACMILLAN and B K SIESJO The effect of hypercapnic acidosis upon the energy metabolism of the brain in arterial hypotension caused by bleeding *Acta phys ol scand* 1973 b *87* 1—14

FREYGANG W H and L SOKOLOFF Quantitative measurement of regional circulation in the central nervous system by the use of radioactive inert gas *Advanc biol med Phys* 1958 *6* 263—279

KENNEDY C Personal communication 1972

KETY S S Theory and applications of the exchange of inert gas at the lungs and tissues *Pharmacol Rev* 1951 *3* 1—41

KETY S S Measurement of local blood flow by the exchange of an inert, diffusible substance In *Methods in medical research* Ed H D Bruner Chicago Yearbook, 1960 *8* 228—236

LANDAU W M W H FREYGANG JR. L. P ROWLAND L SOKOLOFF and S S KETY The local circulation of the living brain values in the unanesthetized and anesthetized cat *Trans Amer neurol Ass* 1955 *80* 125—129

LINDENBERG R Patterns of CNS vulnerability in acute hypoxemia, including anesthesia accidents In *Selective vulnerability of the brain in hypoxemia* Ed J P Schade and W H McMeneme Blackwell Scientific Publ Oxford 1963 189—209

REIVICH M J JEHLE L SOKOLOFF and S S KETY Measurement of regional cerebral blood flow with antipyrine [1] C in awake cats *J appl physiol* 1969 *27* 296—300

Acta physiol. scand. 1973. 87. 78—83

From the Institute of Physiology, University of Lund, Sweden

Choline Acetyltransferase and Cholinesterase in the Pancreatic Duct of the Cat

By

JÖRGEN EKSTRÖM and SVEN LENNINGER

Received 14 April 1972

Abstract

EKSTRÖM, J. and S. LENNINGER. *Choline acetyltransferase and cholinesterase in the pancreatic duct of the cat.* Acta physiol. scand. 1973. 87. 78—83.

Choline acetyltransferase has been demonstrated in the duct and parenchyma of the pancreatic gland of the cat. Potentiation of the effect of acetylcholine on the contraction of ductal segment has been observed after eserinization indicating the presence of cholinesterase in the duct. These findings are considered to indicate that the pancreatic duct of the cat is cholinergically innervated.

The pancreatic duct of the cat develops active tension when suspended longitudinally in oxygenated Krebs solutions of 37° C and contracts when acetylcholine is added to the bath (Lenninger 1972). This finding raises the question if contractions occur also under physiological conditions and if so how they are regulated. Cholinergic nerves have been found in connection with the pancreatic duct of the cat (Richins 1945, Watari 1968), but whether these nerves are functionally innervating the duct is not known. In the present experiments two methods have been used to elucidate this matter. Firstly, the presence of the enzyme choline acetyltransferase in the pancreatic duct and parenchyma of the cat has been shown by its ability to synthesize acetylcholine and secondly, the acetylcholine-splitting enzyme cholinesterase in the duct has been demonstrated by potentiation of the contractile response to acetylcholine by eserine.

Methods

Preparation of duct and gland and assay of choline acetyltransferase

The main pancreatic duct was taken from 23 cats which had been used for other experiments before they were killed. The pancreatic glands were cut free from duodenum and other adjacent structures. The parenchyma of the glands was split bluntly and a crude ductal preparation, about 5 cm (? mg), was obtained from each gland. The ducts were further prepared under dissecting microscope and care was taken to remove all glandular tissue. The wet weights of the ducts thus prepared varied between 1?.0 and 51.2 mg. To obtain enough material for the enzyme assays 10, 7 and 6 ducts, respectively, were pooled.

The choline acetyltransferase activity was determined according to the method of Hebb (see Nordenfelt 1963) The acetone powder of the ducts was made up in cysteine-saline in a concentration of 25 mg/ml of this extract 0 4 ml was incubated at 38 C for 1 h The activity was estimated on the frog rectus The total activity is expressed in µg ACh/h/pooled ducts and the concentration in µg ACh/h/g acetone powder

The enzyme activity was also determined in 5 pancreatic glands from which the main duct had been removed The wet weight of these glands varied between 2 6 and 6 1 g The same procedure was employed in the assay of choline acetyltransferase in the glands as described above with the exception that the extract was made up in cysteine saline in a concentration of 50 mg/ml The activity is expressed in µg ACh/h/gland and the concentration in µg ACh/h/g acetone powder

Preparing of ductal segments and recording of ductal motility

One segment of the pancreatic duct from each of 7 cats were used for these experiments The cats were anesthetized with ether followed by chloralose (80 mg/kg) and the pancreatic gland was exposed through a midline abdominal incision To prevent reflux of intestinal or choledochal material into the pancreatic duct this was ligated where it enters the duodenal wall The cats were killed by air embolus and the pancreatic gland removed The main duct was immediately exposed by blunt dissection about half way between the head and tail of the gland and a piece of about 7 mm length was cut out and placed in Krebs solution of room temperature The segments were further cleansed from glandular tissue under a dissecting microscope and were then arranged in a bath containing o xygenated Krebs solution of 37 C as previously described (Jenninger 1972) A passive tension of 500 dyn was applied to the preparation and the tension was transduced to an ink writing polygraph The continuous tension and the one minute integrated tension were recorded

The following drugs were used Acetylcholine chloride and eserine sulphate The concentrations are expressed in g/ml and refer to the salts

Results

Choline acetyltransferase activity in the pancreatic duct and parenchyma

The total enzyme activity in the three groups of pooled ducts was 4 3 3 7 and 3 0 µg ACh/h respectively This corresponds to a mean total activity of 0 48 µg ACh/h per duct The concentration of choline acetyltransferase in the three groups of pooled ducts was 100 100 and 90 µg ACh/h/g acetone powder respectively (mean and S E 96 6±3 4) The total enzyme activity in the 5 glands was 208 8 137 3 87 5 180 5 and 125 7 µg ACh/h respectively (mean and S E 148 0±21 2) The corresponding concentrations were 240 150 180 280 and 217 µg ACh/h/g acetone powder (mean and S E 213±23)

The activity of the incubate was destroyed when boiled in alkali Furthermore it caused no contraction of the rectus when gallamine (4×10 g/ml) was present in the bath

Spontaneous and stimulated tension of the pancreatic duct before and after eserinization

All 7 segments developed active tension and spontaneous rhythmicity when mounted in the bath These signs of contractile ability usually appeared already after a few minutes When the pattern of motility had stabilized a priming dose of acetylcholine (10^{5} g/ml) was given in most experiments This caused the tension of the duct to rise rapidly After about 1/2 min the bath was emptied washed repeatedly and filled with fresh Krebs solution The ducts then relaxed and remained immobile

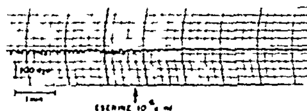


Fig. 1. The effect of eserine on the spontaneous motility of the pancreatic duct

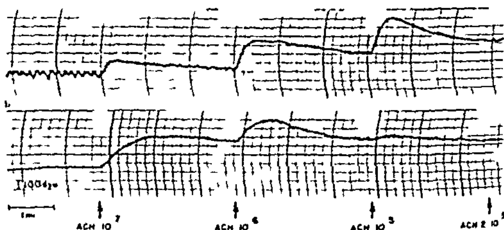


Fig. 2. The responses of a ductal segment to different concentrations of acetylcholine a before and b after eserinization 10^{-6} g/ml

for 2 to 7 min after which time the spontaneous tension and rhythmic contractions reappeared

Acetylcholine was then added to the bath in increasing amounts causing the tension to increase in a typical step-wise manner. The lowest concentration tested was 10^{-7} g/ml and the concentration was then increased by a factor of 10 every third minute until maximal tension was reached. This occurred in one segment at a concentration of 10^{-6} g/ml, in the others at 10^{-5} g/ml. When the concentration was further increased the tension decreased

After completion of the dose response series the bath was emptied and washed. As previously described the duct then relaxed and was left in fresh Krebs solution to regain its activity. When this had become steady eserine sulphate was added to the bath in a concentration of 10^{-6} g/ml. In 5 of the 7 segments a change in the pattern of motility then ensued within a few minutes. Usually the oscillations become smaller than before and in 2 of the segments they disappeared altogether (Fig. 1). The one minute integrated tension increased slightly in 4 of the segments, was unchanged in 2 and decreased in one segment

Between 10 and 15 min after eserinization acetylcholine was added to the bath in the same way as in the previous series. Maximal tension was now reached at a concentration of 10^{-6} g/ml in all segments. Fig. 2 shows the recordings from a typical experiment

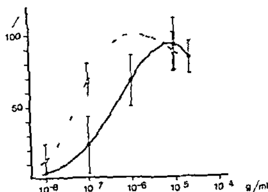


Fig 3 Curves representing the percentual response of the pancreatic duct before (solid line) and after (broken line) eserinization Indicated are the mean responses and the SD of the responses to 5 different concentrations of acetylcholine

To compare the response of the segments to acetylcholine before and after the addition of eserine to the bath the following calculations have been made The highest 1 min integrated tension for each given concentration of acetylcholine was recorded and expressed in absolute arbitrary units From this value the basal 1 min tension was subtracted to obtain the increase in tension caused by the acetylcholine The highest tension calculated for each series was assigned a figure of 100 and the other tensions were expressed in fractions of 100 The figures thus obtained were used to construct individual dose response curves and to estimate the ED_{50} for acetylcholine in each segment before and after eserinization These values are listed in Table I and it can be seen that the mean ED_{50} decreases almost by a factor of 10 after eserinization The difference is significant at the 2 % level using Student's t test for comparison of paired values Fig 3 illustrates the shift to the left of the mean dose response curve after eserinization

Table I The individual and mean values of ED_{50} of acetylcholine before and after eserinization The difference and the significance of the difference between the means are calculated

Cat no	ED_{50} in μg/ml		
	Control		Eserine
1	0 30		0 04
2	0 95		0 08
3	0 35		0 07
4	0 06		0 02
5	0 90		0 08
6	0 25		0 05
7	0 60		0 04
m	0 487		0 054
d		0 434	
S E		0 121	
p		0 02	

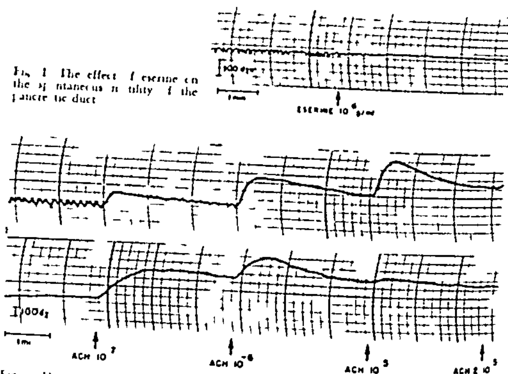


Fig 1 The effect of eserine on the spontaneous motility of the pancreatic duct

Fig 2 The response of a ductal segment to different concentrations of acetylcholine before and after eserinization (10^{-6} g/ml)

for 2 to 7 min after which time the spontaneous tension and rhythmic contractions disappeared

Acetylcholine was then added to the bath in increasing amounts causing the tension to increase in a typical step-wise manner. The lowest concentration tested was $10^{-[illegible]}$ g/ml and the concentration was then increased by a factor of 10 every third minute until maximal tension was reached. This occurred in one segment at a concentration of 10^{-6} g/ml, in the others at 10^{-5} g/ml. When the concentration was further increased the tension decreased.

After completion of the dose response series the bath was emptied and washed. As previously described the duct then relaxed and was left in fresh Krebs solution to regain its activity. When this had become steady eserine sulphate was added to the bath in a concentration of $10^{-[illegible]}$ g/ml. In 5 of the 7 segments a change in the pattern of motility then ensued within a few minutes. Usually the oscillations became smaller than before and in [illegible] of the segments they disappeared altogether (Fig 1). The one minute integrated tension increased slightly in 4 of the segments, was unchanged in 2 and decreased in one segment.

Between 10 and 15 min after eserinization acetylcholine was added to the bath in the same way as in the previous series. Maximal tension was now reached at a concentration of 10^{-6} g/ml in all segments. Fig 2 shows the recordings from a typical experiment.

EKSTROM J Distribution of choline acetyltransferase in the hearts of mammals *Acta physiol scand* 1970 *80* 73—78

GEREBTZOFF M A *Cholinesterases* Pergamon Press London New York Paris Los Angeles 1959 p 138

GRAHAM J D P J D LEVER and T L B SPRIGGS An examination of adrenergic axons around pancreatic arterioles of the cat for the presence of acetylcholinesterase by high resolution autoradiographic and histochemical methods *Brit J Pharmacol* 1968 *33* 15—20

HEBB C O Formation Storage and Liberation of Acetylcholine In *Handbuch der Experimentellen Pharmakologie* XV Springer Verlag Berlin—Gottingen—Heidelberg 1963 55—88

HEBB C and K J HILL Distribution of cholinesterases in the mammalian pancreas *Quart J exp Physiol* 1955 *40* 168—175

HEBB C O and D RATKOVIC Choline acetylase in the placenta of man and other species. *J Physiol* (Lond) 1962 *163* 307—313

LENNINGER S The motility of the pancreatic duct of the cat an in vitro study *Acta physiol scand* 1972 *84* 134—141

NORDENFELT I Choline acetylase in normal and denervated salivary glands. *Quart J exp Physiol* 1963 *48* 67—79

RICHINS C A The innervation of the pancreas *J comp Neurol* 1945 *83* 223—236

WATARI N Fine structure of nervous elements in the pancreas of some vertebrates *Z Zellforsch* 1968 *85* 291—314

Acta physiol. scand. 1973. 87. 84—95

From the Department of Pharmacology, School of Medicine, Linköping, Sweden

Role of Cyclic AMP and Ca^{++} in Mechanical and Metabolic Events in Isometrically Contracting Vascular Smooth Muscle

By

ROLF ANDERSSON

Received 17 April 1972

Abstract

ANDERSSON, R. *Role of cyclic AMP and Ca^{++} in mechanical and metabolic events in isometrically contracting smooth muscle.* Acta physiol. scand. 1973. 87. 84—95.

In bovine mesenteric artery isolated catecholamines stimulated simultaneously adrenergic α- and β-receptors. The relaxation induced by a specific adrenergic β-receptor stimulation was associated with an increased content of cyclic AMP, a phosphorylase a activation and a decrease of the content of ATP and creatine phosphate (CrP). No effect on the high energy phosphate compounds was present in the Ca^{++}-poor artery but the effect on cyclic AMP and phosphorylase was still evident. Sotalol blocked all the actions induced by adrenergic β-receptor stimulation. The contracting action induced by selective adrenergic α-receptor stimulation was initially associated with a decrease of the cyclic AMP content but when the tension was maximally developed after 10 s the cyclic AMP content was increased and the phosphodiesterase activity reduced. In Ca^{++}-poor artery a cyclic AMP decreasing effect was still present after 10 s and there was no reduction of the phosphodiesterase activity. The mechanical and metabolic effects induced by α-receptor stimulation were blocked by dibenamine. Histamine which increased the artery tension significantly reduced the cyclic AMP content after 15 s but after 5 min the level of cyclic AMP had increased and phosphorylase a was activated. The mechanical and metabolic effects of histamine were blocked by promethazine, an antihistaminic drug. The role of cyclic AMP in relaxation and contraction of vascular smooth muscle is discussed.

Catecholamines produce both a vasoconstricting and a vasodilating effect depending on the concentration and vascular region. The vasoconstricting action is by definition exerted by stimulation of adrenergic α-receptors and the vasodilating effect by adrenergic β-receptors (Ahlquist 1948). The vasodilating effect is brought about partly by a direct relaxing action on the vascular smooth muscle and partly by metabolic actions produced by the catecholamines in the surrounding tissue especially the skeletal muscle (Lundholm, Mohme-Lundholm and Svedmyr 1966).

In isolated smooth muscle from rabbit colon a relaxing action was mediated both by adrenergic α- and β-receptors. The metabolic events associated with these kinds of relaxations have been studied (Andersson and Mohme-Lundholm 1969, 1970, Andersson 1972). The relaxing effect exerted by stimulation of adrenergic β-receptors was mediated by cyclic AMP. This signal substance also produced a great

variety of metabolic effects in colonic muscle *viz* activation of phosphorylase *a* an increase in the concentration of carbohydrate metabolites and a reduction of the high energy phosphates (ATP CrP) (Andersson and Mohme Lundholm 1970) Isoprenaline and cyclic AMP produced an increased accumulation of Ca^{++} ions by a microsomal fraction from colonic muscle an effect which was suggested to be the immediate cause of the relaxation (Andersson and Nilsson 1972) The relaxation mediated by the adrenergic *α* receptors was probably dependent on changes in the ionic permeability of the smooth muscle membrane A reduction of the cyclic AMP content was observed after some delay (Andersson and Mohme Lundholm 1970)

The effect of catecholamines and other contracting agents on the lactate and ATP metabolism of vascular and other kinds of smooth muscle have been studied previously (Lundholm and Mohme Lundholm 1962 1965 1966 Mohme Lundholm and Vamos 1967 Andersson Lundholm and Mohme Lundholm 1971) These investigations indicated that catecholamines and Ca stimulated the metabolism in vascular smooth muscle both by the contractile process and by a direct mechanism

In rabbit colon it was demonstrated further that the contractile effect of carbacholine and K ions was accompanied by an increase in the cyclic AMP content This effect was dependent on the presence of Ca^{++} (Andersson 1972 b) It seemed probable however that the increase in cyclic AMP counteracted the contractile action a reduction of the cyclic AMP content by stimulation of the phosphodiesterase activity by imidazole or by cholecystokinin in guinea pig gall bladder was thus accompanied by a contractile action (Andersson *et al* 1972)

In the present investigation the metabolic and mechanical responses associated with selective stimulation of adrenergic *α* and *β* receptors in isolated vascular smooth muscle was studied with special reference to the changes in cyclic AMP The investigation was of interest as the two kinds of adrenergic receptors mediated different mechanical response in this muscle The experiments were performed on isometrically contracting muscle the metabolic effects during isotonic contraction being weaker (Lundholm and Mohme Lundholm 1965)

Methods

The experiments were performed on bovine mesenteric arterie (4—5 mm o d) which consist to about 60—70 % of smooth muscle (Ducret 1930) Each vessel was cut open longitudinally The preparations were 15 mm wide 10—12 mm long and when extended 0 5 mm thick They were mounted in special holders with the muscle fibres running perpendicular to the line of attachment In these holders the distance between the points of attachment of the preparation could be varied and the muscular tension was recorded by a force transducer (FT 03) on a Grass polygraph (Lundholm and Mohme Lundholm 1966) *The preparations were suspended* in 30 ml of Krebs buffer solution with a glucose content of 11 5 mM at 37 C The suspension solution was aerated with 95 % O_2+5 % CO_2 Initially the specimens were lengthened continuously by increasing the tension to 10^4 dyn They reached a length corresponding to about 70 % of the totally relaxed length at a basal tension of 10^4 dyn (Lundholm and Mohme Lundholm 1966)

To reduce the Ca content of the preparations they were suspended in a Ca^{++} free Krebs buffer solution at 4 C to which 0 2 mM EGTA was added The buffer was changed several times during a 24 h period The preparations were then incubated in Ca free Krebs buffer at 37 C but without EGTA After these incubation periods in Ca free buffer the preparation did not respond to contracting agents

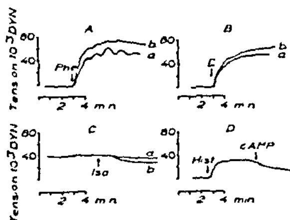


Fig. 1. Bovine mesenteric artery. A. The contracting effect of phenylephrine (Phe 2×10^{-6} g/ml) with (b) and without (a) sotalol (1.2×10^{-5} g/ml). B. The effect of epinephrine (E 5×10^{-7} g/ml) on muscle tension in presence (b) and absence (a) of sotalol (1.2×10^{-5} g/ml). C. The relaxant effect of isoprenaline (Iso 5×10^{-7} g/ml) with (b) and without (a) dibenamine (5×10^{-6} g/ml). D. The relaxant effect of cyclic AMP (1×10^{-3} M) on a histamine (Hist 1×10^{-6} g/ml) contracted muscle.

In order to specifically stimulate adrenergic α-receptors in bovine mesenteric arteries, l-phenylephrine (2×10^{-6} g/ml) was added to a preparation pretreated with sotalol (1.2×10^{-5} g/ml). For stimulating of adrenergic β-receptors l-isoprenaline (5×10^{-7} g/ml) was added to a preparation pretreated with dibenamine (5×10^{-6} g/ml).

To inhibit adrenergic α- or β-receptors, dibenamine and sotalol, respectively, were used in the concentrations mentioned. When studying the relaxing action of β-receptor stimulation, the tension of the muscle preparation was first increased by pretreatment with histamine (1×10^{-6} g/ml).

At the end of each experiment the muscle preparation was quickly frozen in freon 12 containing solid CO_2 at −60 °C. The hexose phosphates, ATP and CrP were analyzed by enzymatic methods as described by Andersson and Mohme-Lundholm (1970); the phosphorylase activity was determined according to Bueding *et al.* (1962), phosphodiesterase according to Kukovetz and Poch (1970) as described by Andersson (1972) and cyclic AMP according to Kakiuchi and Rall (1968).

The samples for calcium analysis were prepared by digestion with ultrapure HNO_3 and Ca was determined by means of a Unicam Atom Spectrophotometer (Parker 1964).

Results

Specific stimulation of adrenergic α- and β-receptors in bovine mesenteric arteries. In smooth muscle from rabbit colon it was possible to induce a specific stimulation of adrenergic α-receptors by submaximal concentrations of l-phenylephrine and of β-receptors by l-isoprenaline (Andersson and Mohme-Lundholm 1969, 1970). In bovine mesenteric arteries it was not possible to stimulate specifically one of the receptors alone by using one of these agonists. Thus, although phenylephrine in a concentration of 2×10^{-6} g/ml markedly increased the tension, the contraction was further increased when the preparation was pretreated with the β-receptor blocking agent sotalol (Fig. 1 A). This compound is devoid of local anesthetic effects (Åberg and Welin 1967), which otherwise may reduce the action of contracting agents in smooth muscle (Åberg and Andersson 1972). In the preparation contracted by histamine, l-isoprenaline (5×10^{-7} g/ml) alone induced a very small relaxing effect. After pretreatment of the muscle with dibenamine (5×10^{-6} g/ml), however, the relaxing effect was larger (Fig. 1 C).

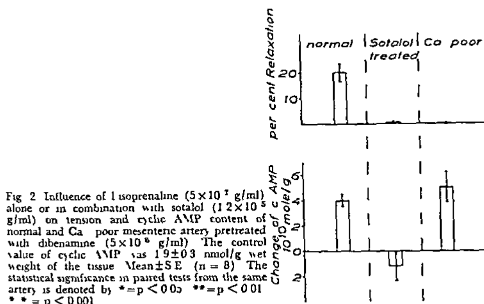


Fig 2 Influence of l isoprenaline (5×10^{-7} g/ml) alone or in combination with sotalol (1.2×10^{-5} g/ml) on tension and cyclic AMP content of normal and Ca poor mesenteric artery pretreated with dibenamine (5×10^{-6} g/ml) The control value of cyclic AMP was 1.9 ± 0.3 nmol/g wet weight of the tissue Mean ± S E (n = 8) The statistical significance in paired tests from the same artery is denoted by * = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

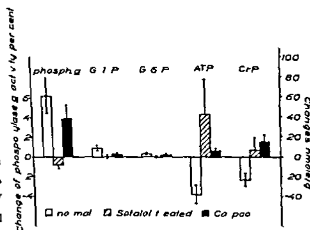


Fig 3 Effects on phosphorylase a activity glucose 1 phosphate glucose 6-phosphate ATP and CrP contents of mesenteric artery treated as described in Fig 2 Mean ± S E (n = 10) Statistical significance as in Fig 2

In order to specifically stimulate adrenergic α or β receptors in mesenteric arteries in the present study a fairly specific agonist was used in combination with an antagonist for blocking the other kind of receptor i e phenylephrine + sotalol and isoprenaline + dibenamine for stimulation of adrenergic α and β receptors respectively That this kind of approach was necessary to evaluate the role of the adrenergic receptors in changes of the cyclic AMP content in vascular smooth muscle was further demonstrated when an agonist (epinephrine) which markedly stim both α and β receptors was used

TABLE I. The activity of phosphorylase a (per cent of total activity) and the content of cyclic AMP, glucose-1-phosphate, ATP, CrP and calcium in normal and Ca-poor bovine mesenteric artery

	c AMP 10 [illegible] mol/g	Phosph. a	G 1 P nmol/g	ATP nmol/g	CrP nmol/g	Ca nmol/kg
Normal muscle	15.3 ± 1.7 n = 24	18.3 ± 2.8 n = 10	25 ± 3 n = 15	430 ± 43 n = 14	161 ± 40 n = 14	12.1 ± 0.38 n = 8
Ca-poor muscle	9.0 ± 1.0 n = 24	8.5 ± 1.7 n = 10	17 ± 2 n = 10	311 ± [illegible]0 n = 10	135 ± 11 n = 9	1.16 ± 0.06 n = 8
Differences	6.3 ± 2.0 p < 0.01	9.8 ± 3.3 p < 0.01	8 ± 4 n.s.	119 ± 66 n.s.	26 ± 41 n.s.	10.94 ± 0.38 p < 0.001

Metabolic effects of adrenergic β receptor stimulation in normal muscle. To achieve selective adrenergic β-receptor stimulation, isoprenaline in a concentration of 5×10^{-7} g/ml was added to the histamine-contracted artery pretreated with 5×10^{-6} g/ml dibenamine in order to block the action of adrenergic α receptors. The isoprenaline induced relaxation became manifest after a latency period of about 20 s and was complete after 2–3 min. The decrease in tension was fairly moderate and amounted to about 20 % of the maximal tension induced by histamine. An increase in the cyclic AMP content was noted after 2 min (Fig. 2). The phosphorylase a activity and the hexose phosphates G-1-P and G-6-P also increased. The contents of ATP and CrP decreased (Fig. 3). When the isoprenaline induced relaxation was blocked by sotalol (1.2×10^{-5} g/ml) the metabolic effects normally appearing after 2 min were also inhibited (Fig. 2 and 3). Sotalol in itself did not have any significant metabolic effects.

Metabolic effects in Ca-poor mesenteric arteries. When the mesenteric arteries were suspended in a Ca-free Krebs solution to which EGTA was added as described in Methods, the Ca content decreased to 1/10 of that in the normal muscle (Table I). In these Ca-poor muscles neither histamine nor phenylephrine had any contracting action but after addition of Ca to these muscles this action was restored. The reduction of the Ca content of the muscle was accompanied by a significant decrease of the basal cyclic AMP content and a reduction of the phosphorylase a activity (Table I).

In the Ca-poor muscle isoprenaline increased the cyclic AMP content to the same degree as in the normal muscle (Fig. 2). The stimulating effect on the phosphorylase a activity was still present (Fig. 3). Isoprenaline had no decreasing action on the ATP and CrP contents, however (Fig. 3).

Relaxing and metabolic actions of cyclic AMP in mesenteric arteries. A comparison between the relaxing effects of cyclic AMP and isoprenaline showed that cyclic AMP

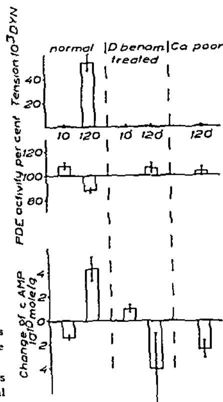


Fig 4 The effects of l phenylephrine (2×10^{-6} g/ml) alone or in combination with dibenamine (5×10^{-6} g/ml) on tension phosphodiesterase activity and cyclic AMP content of normal and Ca poor artery pretreated with sotalol (1.2×10^{-5} g/ml) Mean ± S E (n = 10—12) Statistical significance as in Fig 2

in a concentration of 1×10^{-3} M induced relaxation of almost equal magnitude as that induced by isoprenaline (5×10^{-8} g/ml) (Fig 1 D) At this concentration cyclic AMP also increased the phosphorylase *a* activity significantly After 2 min there was an increase in the phosphorylase *a* activity by 5 5 ± 1 3 % from a control value of 15 4 ± 2 7 % (n = 5 $p < 0.01$) The ATP content decreased from a basal value of 829 ± 87 nmol/g by 81 ± 18 nmol/g in paired tests (n = 7 $p < 0.01$)

Mechanical and metabolic effects associated with adrenergic α receptor stimulation In order to obtain selective adrenergic *α* receptor stimulation l phenylephrine in a concentration of 2×10^{-6} g/ml was added to muscle preparations pretreated with 1.2×10^{-5} g/ml sotalol to block any *β* receptor stimulation The tension of the *β* receptor blocked muscle increased more than that of a muscle not pretreated by sotalol

After the addition of l phenylephrine + sotalol a biphasic change of the cyclic AMP content was observed After 10 s just before the tension had started to rise it showed a decrease and after 120 s when the contraction was complete it was found to have increased (Fig 4) As regards the phosphodiesterase activity an inhibition of this enzyme was observed after 120 s (Fig 4) The increase in cyclic

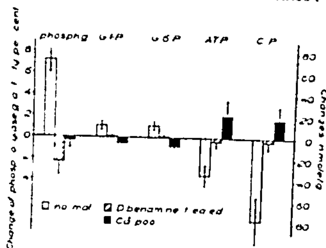


Fig 5 Changes of phosphorylase *a* activity glucose 1 phosphate glucose 6 phosphate ATP and CrP contents in mesenteric artery after treatment as mentioned in Fig 4 Mean±S E (n = 10) Statistical significance as in Fig 2

AMP content was accompanied by an increase in the phosphorylase *a* activity and hexose phosphate contents (Fig 5) As described earlier by Beviz *et al* (1965) the development of the tension was associated with a reduction of the ATP and CrP contents (Fig 5)

The adrenergic α blocking agent dibenamine (5×10^{-6}) which in itself had no effect on the metabolism or muscle tension blocked all the actions produced by phenylephrine after 2 min (Fig 4 and 5) The decrease of the cyclic AMP content observed after 10 sec was blocked by dibenamine (Fig 4)

The metabolic changes associated with contraction in rabbit colonic muscle were Ca dependent (Andersson 1972) There was reason to believe that the same relationship applied in mesenteric artery In the Ca poor sotalol treated mesenteric artery a decrease of cyclic AMP content was still evident 2 min after addition of phenylephrine The decrease was of the same magnitude as after 10 s in the normal preparation (Fig 4) There was also indication of a decreased glycogenolysis in the Ca poor preparation as the contents of G 6 P and F 6 P were reduced (Fig 5) It was thus evident that the increase in the cyclic AMP content following contraction was Ca dependent

Metabolic actions of epinephrine in normal and Ca poor muscle Epinephrine stimulates both α and β receptors and it was of interest to study the effects of catecholamine derivative with this double action The influence of epinephrine (5×10 [illegible] g/ml) on the tension and cyclic AMP content was studied 2 min after its addition to mesenteric arteries Epinephrine increased the tension of the mesenteric artery to about 3×10^{4} dyn There was also an increase of the cyclic AMP content (Fig 6)

When the muscle was pretreated by sotalol (1.2×10^{-5} g/ml) the contractile effect of epinephrine was potentiated (Fig 1 B) and the cyclic AMP content still increased

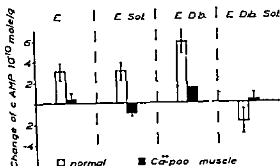


Fig 6 Influence of epinephrine (5×10^{-7} g/ml) alone or in combination with sotalol (1.2×10^{-5} g/ml) and /or dibenamine (5×10^{-6} g/ml) on the cyclic AMP content of mesenteric artery 2 min after addition of stimulating drug Mean±S E (n = 5—7) Statistical significance as in Fig 2

(Fig 6) When the adrenergic α receptors were blocked by dibenamine (5×10^{-6} g/ml) the contractile effect of epinephrine was eliminated its cyclic AMP increasing effect persisted This effect was blocked only by a combination of sotalol and dibenamine (Fig 6)

The effects of epinephrine on the cyclic AMP content was also studied in the Ca^{++} poor muscle Here epinephrine had no effect on the cyclic AMP content In combination with sotalol or dibenamine an effect on this content was observed however When the Ca^{++} poor muscle was pretreated with sotalol there was a decrease of the cyclic AMP content of almost the same magnitude as after phenylephrine (Fig 6) In the Ca^{++} poor muscle pretreated with dibenamine epinephrine increased the content of cyclic AMP (Fig 6) It seems as though an increasing action of β receptor stimulation on the cyclic AMP content was balanced by a decreasing action of α stimulation There was no effect of epinephrine on the cyclic AMP content in the Ca^{++} poor muscle pretreated by a combination of sotalol and dibenamine (Fig 6)

Blockade of the mechanical and metabolic effects of histamine Histamine showed the same biphasic change of the cyclic AMP content as phenylephrine did After 15 s histamine had reduced the cyclic AMP content with $2.0\pm0.09\times10^{-10}$ mol/g ($p<0.05$) When histamine (1×10^{-6} g/ml) had induced a maximal contraction the phosphodiesterase activity was inhibited to about 85 % of the control value there was an increase of the cyclic AMP content after 5 min and the phosphorylase *a* activity was increased too (Fig 7) In the Ca^{++} poor muscle where histamine did not produce any tension the metabolic effects were also abolished

Promethazine (4.2×10^{-6} g/ml) an antihistaminic drug reduced the tension of the mesenteric artery but had no metabolic effects of its own It blocked the mechanical and metabolic actions produced by histamine (Fig 7)

Discussion

In this study the action of the catecholamines on the cyclic AMP content of the smooth muscle of the bovine mesenteric artery was found to be the result of at least 3 different components which are discussed in the following

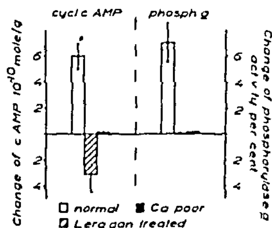


Fig 7 Changes in the cyclic AMP content and phosphorylase *a* activity 5 min after addition of histamine (1×10^{-6} g/ml) to normal, Ca poor or promethazine (Lergigan®) (4×10^{-6} g/ml) treated mesenteric artery. Mean ± S.E. (n = 6–8). Statistical significance as in Fig

Selective stimulation of the adrenergic β receptors resulted in an increased cyclic AMP content. This increase was accompanied by an increase in the phosphorylase *a* activity and in the contents of hexose phosphates but a decrease in the content of the high energy phosphates ATP and CrP. The mechanical and metabolic effects were blocked by an adrenergic β receptor blocking agent. In the Ca poor muscle β receptor stimulation still increased the cyclic AMP content and the phosphorylase *a* activity, no reduction of the ATP and CrP contents occurred, however.

In the mesenteric artery adrenergic β receptor mediated relaxation was associated with changes of the same metabolic parameters as in rabbit colonic muscle (Andersson and Mohme Lundholm 1970, Andersson 1972). Exogenously supplied cyclic AMP relaxed the bovine mesenteric artery and the relaxation was associated with the same metabolic changes as produced by adrenergic β receptor stimulation. These findings indicate a relationship in vascular smooth muscle between adrenergic β-receptor mediated relaxation and an increased cyclic AMP content.

In skeletal and cardiac muscle the relaxation—contraction cycle is dependent on the myoplasmic concentration of free Ca (Sandow 1965, Ebashi and Endo 1968, Ruegg 1971). In the sarcoplasmic reticulum of the heart catecholamines have been suggested to play an important role in the regulation of the myoplasmic Ca concentration (Entman *et al* 1969).

Smooth muscle cells contain only a small amount of sarcoplasmic reticulum as compared with the skeletal and cardiac muscles. In the vicinity of this structure several mitochondria have been observed suggesting that the cellular metabolism is very active at these places (Rhodin 1962, Nagasawa and Suzuki 1967). Andersson and Nilsson (1972) isolated a microsomal fraction from rabbit colon which accumulated Ca in the presence of ATP and Mg. This effect was stimulated by isoprenaline and cyclic AMP, the former action was blocked by sotalol. It was therefore suggested that the relaxation produced by stimulation of adrenergic β receptors was mediated by cyclic AMP which stimulated a Ca^{++} accumulating ATP utilizing mechanism which reduced the myoplasmic Ca concentration. Simultaneously the

glycogenolysis is stimulated possibly to provide energy for these cell functions In vascular smooth muscle stimulation of adrenergic β receptors resulted in a reduction of the ATP and CrP contents effects which were dependent on the presence of Ca^{++} It seems fairly probable therefore that a Ca binding mechanism exists in vascular smooth muscle also

It was originally suggested by Robison Butcher and Sutherland (1967) that adrenergic α receptor stimulation was associated with a reduction of the adenyl cyclase activity Evidence from different kinds of tissues supporting this hypothesis has sinse been reviewed by Robison and Sutherland (1970) In vascular smooth muscle in the present study selective α receptor stimulation was associated with an initial reduction of the cyclic AMP content (after 10 s) and in the absence of Ca^{++} ions this reduction still persisted after 2 min In isotonically contracting vascular smooth muscle Volicer and Hynie (1971) found that norepinephrine and angiotensin decreased the incorporation of ^{3}H adenosine in cyclic AMP This evidence indicates that adrenergic α receptor stimulation both in vascular smooth muscle and other tissues is associated with a reduced cyclic AMP content

In the presence of Ca ions the reduction of the cyclic AMP content in vascular smooth muscle was however later masked by an increasing action associated with a reduction of the phosphodiesterase activity (Andersson 1972) In the Ca^{++} poor mesenteric artery the inhibition of the phosphodiesterase activity and the increase of cyclic AMP content produced both by adrenergic α receptors stimulation and histamine was lost

The initial reducing action of the adrenergic α receptor stimulation on the cyclic AMP content was shared with that of histamine In earlier studies on rabbit colon it was found that carbacholine and K ions initially reduced the cyclic AMP content an effect that was more persistant in the Ca^{++} poor muscle (Andersson 1972) In the isolated guinea pig gall bladder cholecystokinin contracted the muscle and reduced the cyclic AMP content by activating phospodiesterase (Andersson *et al* 1972)

A reduction of the cyclic AMP content of smooth muscle seems therefore to be an initial event associated with the contractile process As cyclic AMP stimulated the binding of Ca^{++} by a microsomal fraction of smooth muscle (Andersson and Nilsson 1972) a reduction of the cyclic AMP content will probably lead to a release of Ca^{++} from this fraction and a contractile action might be induced We have recently found that carbacholine increases the Ca^{++} release from the Ca^{++} binding fraction of intestinal smooth muscle (Andersson and Nilsson unpublished) An objection to this hypothesis is however that the cyclic AMP content increases secondarily as the phosphodiesterase activity is inhibited by the released Ca^{++} If the hypothesis is correct one must therefore assume that cyclic AMP is compartmentilized in the smooth muscle cell one fraction of cyclic AMP regulating the Ca binding and another one the phosphorylase *a* activity and possibly other metabolic functions There is evidence of compartmentilization of cyclic AMP in rabbit colon (Andersson *et al* 1972) but much more evidence is needed to sustain this hypothesis

I am indebted to the assistance of Miss Marianne Borg, Mrs Ingrid Salomonsson and Mr Sverker Borjesson. Financial support has been provided by the Swedish Medical Research Council (B 0-14X 060-01A and B 0-14X 101-07B).

References

ABERG, G. and R. ANDERSSON: Studies on mechanical actions of mepivacaine (Carbocaine®) and its optically active isomers in isolated smooth muscle: role of Ca and cyclic AMP. *Acta pharmacol. (Kbh.)* 1972. In press.
ABERG, G. and I. WELIN: Interaction of beta receptor blocking agents with the nerve blocking effects of procaine in vitro. *Life Sci.* 1967, 6, 975—983.
AHLQUIST, R. P.: A study of the adrenotropic receptors. *Amer. J. Physiol.* 1948, 153, 586—600.
ANDERSSON, K. F., R. ANDERSSON and P. HEDNER: Cholecystokinetic effect and concentration of cyclic AMP in gallbladder muscle in vitro. *Acta physiol. scand.* 1972, 85, 511—516.
ANDERSSON, R.: Role of cyclic AMP and Ca in the metabolic and relaxing effects of catecholamines in intestinal smooth muscle. *Acta physiol. scand.* 1972, 85, 312—322.
ANDERSSON, R.: Relationship between cyclic AMP, Ca, phosphodiesterase activity and contraction in smooth muscle. *Acta physiol. scand.* 1973. In press.
ANDERSSON, R. and E. MOHME-LUNDHOLM: Studies on the relaxing actions mediated by stimulation of adrenergic α- and β-receptors in taenia coli of the rabbit and guinea pig. *Acta physiol. scand.* 1969, 77, 372—384.
ANDERSSON, R. and E. MOHME-LUNDHOLM: Metabolic actions in intestinal smooth muscle associated with relaxation mediated by adrenergic α- and β-receptors. *Acta physiol. scand.* 1970, 79, 244—261.
ANDERSSON, R. and K. NILSSON: Relaxation in intestinal smooth muscle: role of cyclic AMP and calcium. *Nature (Lond.)* 1972, 238, 119—120.
ANDERSSON, R., L. LUNDHOLM, L. and E. MOHME-LUNDHOLM: Relationship between mechanical and metabolic events in vascular smooth muscle. *Proc. Symp. Physiol. Pharmacol. Vasc. Neuroeffector Systems.* S. Karger, Basel 1971, 20—215.
ANDERSSON, R., L. LUNDHOLM, E. MOHME-LUNDHOLM and K. NILSSON: Evidence of compartmentalization of cyclic AMP in smooth muscle. *Acta physiol. scand.* 1973. In press.
BENTZ, A., L. LUNDHOLM, E. MOHME-LUNDHOLM and N. VAMOS: Hydrolysis of adenosinetriphosphate and creatinephosphate on isometric contraction of vascular smooth muscle. *Acta physiol. scand.* 1965, 65, 268—272.
BUEDING, E., E. BULBRING, H. KURIYAMA and G. GERCKEN: Lack of activation of phosphorylase by adrenaline during its physiological effect on intestinal smooth muscle. *Nature (Lond.)* 1962, 196, 944—946.
BURGRET, S.: Die Dehnungseigenschaften der Coronar und Splanchnicus Arterien. *Pflügers Arch. ges. Physiol.* 1930, 225, 669—680.
EBASHI, S. and M. ENDO: Calcium ion and muscle contraction. *Progr. Biophys. molec. Biol.* 1968, 18, 123—183.
EXTON, M. J., C. L. PARK and S. E. EISTEIN: Mechanism of action of epinephrine and glucagon on the canine heart. *Circulat. Res.* 1969, 25, 429—438.
KAKIUCHI, S. and T. W. RALL: The influence of chemical agents on the accumulation of adenosine 3',5'-phosphate in slices of rabbit cerebellum. *Molec. Pharmacol.* 1968, 4, 367—378.
KUKOVETZ, W. R. and G. POCH: Inhibition of cyclic 3',5'-nucleotide-phosphodiesterase as a possible mode of action of papaverine and similarly acting drugs. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmacol.* 1970, 267, 189—194.
LUNDHOLM, L. and E. MOHME-LUNDHOLM: Studies on the effects of drugs upon the lactic acid metabolism and contraction of vascular smooth muscle. *Acta physiol. scand.* 1960, 53, 45—63.
LUNDHOLM, L. and E. MOHME-LUNDHOLM: Energetics of isometric and isotonic contraction in isolated vascular smooth muscle under anaerobic conditions. *Acta physiol. scand.* 1965, 64, 275—282.
LUNDHOLM, L. and E. MOHME-LUNDHOLM: Length at inactivated contractile elements, length-tension diagram, active state and tone of vascular smooth muscle. *Acta physiol. scand.* 1966, 68, 347—359.
LUNDHOLM, L., E. MOHME-LUNDHOLM and N. SVEDMYR: Physiological Interrelationships. Introductory remarks. *Pharmacol. Rev.* 1966, 18, 255—272.
MOHME-LUNDHOLM, E. and N. VAMOS: Influence of Ca^{++} deficiency and pronethalol on metabolic and contractile effects of catecholamines and K ions in vascular muscle. *Acta pharmacol. (Kbh.)* 1967, 25, 87—96.

NAGASAWA J and SUZUKI T Electron microscopic study on the cellular interrelationships in smooth muscle *Tohuku J exp Med* 1967 *91* 299—313
PARKER H E Magnesium calcium and zink in animal nutrition *Atomic Absorption News letter* 1964 No 13 1—7
RHODIN J A G Fine structure of vascular walls in mammals *Physiol Rev* 1962 *42* 48—87
ROBISON G A R W BUTCHER and E W SUTHERLAND Adenyl cyclase as an adrenergic receptor *Ann N Y Acad Sci* 1967 *139* 703—723
ROBISON G A and E W SUTHERLAND Sympathin E Sympathin I and the intracellular level of cyclic AMP *Circulat Res* 1970 26 Suppl I 147—161
RUEGG J C Smooth muscle tone *Physiol Rev* 1971 *51* 201—248
SALUKHE P V and N S DHALLA Excitation contraction coupling in heart evidence against the involvement of adenosine cyclic 3 5 monophosphate in calcium tran port by sarcotubular vesicles of canine myocardium *Molec Pharmacol* 1970 6 659—666
SANDOW A Excitation—contraction coupling in skeletal muscle *Pharmacol Rev* 1965 *17* 265—320
VOLICER L and HYNIE S Effect of catecholamines and angiotensin on cyclic AMP in rat aorta and tail artery *Europ J Pharmacol* 1971 *15* 214—220

Acta physiol. scand. 1973. 87. 96—102

From the Institute of Neurophysiology, University of Oslo, Norway

EEG Synchronization and Sleep Induced by Stimulation of the Medial and Orbital Frontal Cortex in Cat

By

E. ALNÆS, B. R. KAADA and K. WESTER

Received 19 April 1972

Abstract

ALNÆS, E., B. R. KAADA and K. WESTER. *EEG synchronization and sleep induced by stimulation of the medial and orbital frontal cortex in cat.* Acta physiol. scand. 1973. 87. 96—102.

Electrocortical synchronization associated behaviourally with light sleep was induced by low frequency stimulation of the subcallosal region and the orbital gyrus of the frontal lobe in unanesthetized cats. The effect was weaker than that obtained with basal forebrain stimulation. The above cortical areas have previously been shown to exert strong inhibitory influences on a variety of somatomotor and autonomic activities. High frequency stimulation of the same areas produces electrocortical desynchronization and behavioural arousal. It is suggested that the subcallosal and orbital portions of the frontal cortex give origin to a descending system capable of modulating the excitatory state of sleep-producing mechanisms in the brain stem or diencephalon.

Behavioural sleep associated with synchronization of the electrocortical activity has previously been induced by *low* frequency stimulation (5—12/s) of various *subcortical* areas. These areas appear to be parts of a continuous core of the brain stem and ventral diencephalon possessing similar functional properties. This core includes the intralaminar thalamic nuclei, the centre median de Luys and nucleus ventralis anterior (Hess 1944, Akert, Koella and Hess Jr 1952), the pontine and mesencephalic reticular formation (Favale *et al.* 1961) and the preoptic region (Sterman and Clemente 1962, Clemente and Sterman 1963, Hernandez Peon and Ibarra 1963). Similar effects have been obtained by low frequency stimulation of afferent nerves, i.e. the somatic cutaneous group II (Pompeiano and Swett 1962) and the baroceptors from the carotid sinus (Bonvallet *et al.* 1954). From these studies the idea of an ascending deactivating and inhibitory system developed, a counterpart of the ascending activating system of Moruzzi and Magoun (1949).

EEG synchronization has also been elicited by low frequency stimulation of the amygdaloid nuclear complex (Clemente and Sterman 1963, Hernandez Peon and Ibarra 1963) and caudate nucleus (Akert and Andersson 1951, Stevens, Kim and McLean 1961).

High frequency stimulation (30—300/s) of all these subcortical areas produces the opposite effect behavioural arousal associated with electrocortical desynchronization This has been shown for the reticular formation (Moruzzi and Magoun 1949, Favale *et al* 1961) the hypothalamus and preoptic region (Murphy and Gellhorn 1945 Hernandez Peon and Ibarra 1963) the amygdala (Kaada 1951 Ursin and Kaada 1960 Hernandez Peon and Ibarra 1963) and caudate nucleus (Gerebtzoff 1941 Shimamoto and Verzeano 1954 Stoupel and Terzuolo 1954)

Widespread electrocortical desynchronization may also be evoked on high frequency stimulation of certain *cortical* fields on the medial ventral and lateral aspects of the hemisphere (Kaada 1951 Segundo *et al* 1955, Kaada and Johannessen 1960) The behavioural correlate to this response is in the unanesthetized animal a typical arousal and orienting response (Segundo *et al* 1955 Fangel and Kaada 1960)

The purpose of the present study was to stimulate two of these cortical zones in unanesthetized animals through chronically implanted electrodes to see whether low frequency pulses could also reverse the responses to cortical stimulation to one of synchronization and behaviourally resulting in sleep A previous report has indicated that electrocortical synchronization can occur on stimulation of the medial and orbital regions (Kaada 1951 pp 234 and 245) but no systematic study has been carried out attempting to correlate the electrocorticographic and behavioural responses A preliminary report concerning the subcallosal region has been given (Kaada Wester and Alnæs 1965)

Methods

Either 2 or 3 bipolar concentric needle electrodes were implanted under aseptic precautions in 17 adult tame cats under Nembutal® (Abbott) anesthesia in the medial frontal cortex 0 5 to 1 5 mm from the midline and in the orbital gyrus and its surroundings Electrodes were also placed in the preoptic region for comparison of the effects obtained from cortical and subcortical areas The cannula tubing and its two inside wires (100 μm) were insulated except for their tips The distance between the two electrode tips was 1 mm The electrodes could be moved in a vertical direction through holes in a 6 mm high metal bracket screwed onto the dorsal surface of the skull They could be fixed in any vertical position by a screw through the bracket thus allowing stimulation in the awake animal of any desired point along each electrode track In 4 animals the lateral frontal cortex was exposed and 4 to 6 flexible wires (250 μm) (insulated except at their tips) were placed on the orbital cortex They were fixed to the skull by acrylic cement and drawn subcutaneously to Winchester microplugs fastened to a collar A square wave stimulator was used and the stimulus parameters employed varied from 5—300/s 0 5—3 ms duration and 0 08—0 48 mA (1—6 V)

Electrocorticograms were recorded bipolarly from 4 cortical areas 2 pairs of electrodes being symmetrically placed over the right and left lateral frontal regions and 2 over the parieto-occipital areas Small steel needles were driven into the skull and soldered to screened thin wires drawn subcutaneously to the microplugs on the collar through which the electrodes were connected to an 8 channel EEG apparatus (Ediswan Mark II) The animals were kept in a semi soundproof observation cage with a glass window Stimulation was carried out in the freely moving animal 2 to 6 days postoperatively

At termination of the experiment electrolytic lesions were made at the electrode tips and the brains were fixed in 10 formalin The location of most electrode tips could be identified by inspection of the lateral and medial surface of the brain When necessary serial sections (20 m thick) were made every 20th section being stained according to the Nissl method

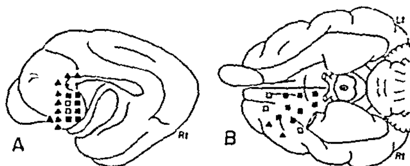


Fig. 1. Diagrammatic representations of (A) medial and (B) ventral views of the cat brain showing the location of electrode placements in the medial cortex (A) and orbital cortex (B). Right olfactory bulb has been deleted for clarity. *Squares* indicate points which on low frequency stimulation (6/sec) induced EEG synchronization and behavioural sleep. Open squares: weak effects; solid squares: stronger effects. High frequency stimulation of the same points produced desynchronization and behavioural arousal.—*Triangles*: points from which EEG desynchronization and behavioural arousal were induced by high frequency stimulation (300/sec) but with no effects on low frequency stimulation. *Dots*: no EEG or behavioural responses.

Results

High frequency stimulation (30—300/s) of the points indicated by triangles and open and filled squares in Fig. 1 A and B consistently produced behavioural arousal associated with electrocortical desynchronization. Behavioural arousal was typified by the following sequence of events. Stimulation resulted in arrest of all ongoing activity during which time the animal appeared alert and attentive. The cat would then raise its head and perform searching movements, usually towards the contralateral side. This response and the accompanying desynchronization outlasted the stimulation for 15—60 s or more (Fig. 2 A—B).

During such poststimulatory periods of desynchronization a 6/s stimulus of 10—15 s duration was applied at intervals of 6—12 s. During this low frequency stimulation the desynchronization was frequently replaced by 6—12/s bursts of spindle activity and slow waves (Fig. 2 C—D). Such synchronization was produced when stimulating the points indicated by open or filled squares in Fig. 1, the best effects being obtained from the filled squares. Following three or more such stimulations the animal often appeared drowsy and would lay down and close its eyes in preparation for sleep. The cat could readily be awakened by auditory stimulation or by applying a high frequency stimulus through the same cortical electrode. Neither deep sleep (with dominance of delta waves in the EEG) nor paradoxical sleep (behavioural sleep associated with desynchronization) occurred as a result of cortical stimulation.

To induce light sleep with electrocortical synchronization it was necessary that the animal not be too active and alert, but in a quiet and resting state. In 2 cats which were very active and aroused during the entire experiment no effects of

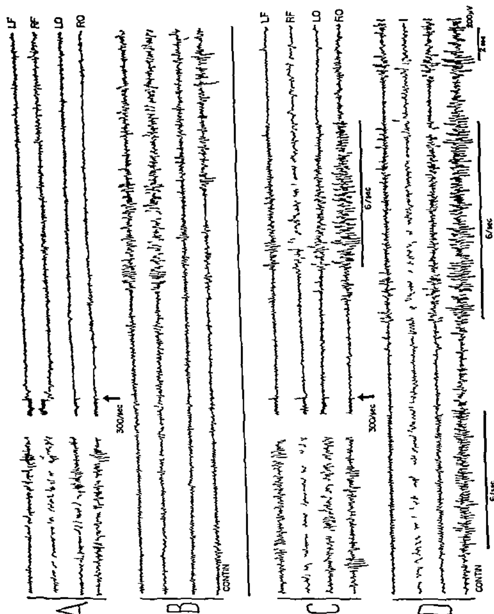


Fig 2 *A* left EEG before stimulation Right desynchronization resulting from high frequency stimulation of the subcallosal cortex (300/s 0 18 mA 1 ms 10 s) Arrow indicates termination of the stimulus artifact *B* continuation of record *A* — *C—D* Same as in *A—B* except that high frequency stimulation (terminated at arrow in C) is now followed by intermittent low frequency stimulation (6/s 0 18 mA 1 ms) of the same cortical point

partial effects were obtained with respect to the EEG and behaviour. It appears as if the animal has to be in a relaxed state before these effects can be induced. Similar observations have been made in stimulating subcortical synchronizing areas (Favale *et al.* 1961). Noisy surroundings similarly prevented the occurrence of sleep.

The cortically induced synchronization was frequently compared with that elicited by stimulation of the basal forebrain in the preoptic region. It should be emphasized that on the whole the cortically induced effects were weaker (higher stimulus threshold) and more inconsistent compared to the responses produced from the preoptic region. The stimulus threshold for obtaining synchronization on cortical stimulation was about twice that required on stimulation of the most effective preoptic sites: 0.16—0.32 mA and 0.08—0.16 mA, respectively. However, when clearly present, the cortically evoked effects could readily be reproduced over a period of several days.

The best synchronization effects with cortical stimulation were obtained from the posterior part of the subcallosal region and the orbital cortex (Fig. 1 A and B, filled squares). In the adjacent areas, the response was weaker (open squares). From the pre- and supracallosal region no clear-cut synchronization could be induced, whereas desynchronization at high frequency stimulation was produced from the sites indicated by triangles in Fig. 1.

It has previously been demonstrated that the various somatomotor and autonomic responses which can be induced by stimulating the subcallosal and orbital regions are of cortical origin, since they are largely diminished by cortical application of local anesthetics (Kaada 1951; Setekleiv 1964; Gjone 1966). Similarly, in the present study the electrocortical synchronization in response to stimulation was greatly reduced following electrocoagulation of the cortical point stimulated. Therefore the effects were not due to spread of current to the ventral diencephalon or the caudate nucleus. Further, it has been demonstrated by Laursen (1963) that the electrocortical synchronization previously described as a result of caudate stimulation might be due to spread of current to the internal capsule or the thalamus. The latter structures, however, are situated at a great distance from the electrode tip in the subcallosal region.

Discussion

The data presented demonstrate that electrocortical synchronization and light sleep may be induced not only by subcortical stimulation, but also from the cerebral cortex. The latter effect is most likely mediated through the sleep-producing mechanisms of the brain stem and diencephalon by way of fiber connections known to exist between the subcallosal and orbital cortex and these subcortical areas. The evidence for such cortico-subcortical routes for the inhibitory motor and visceral effects produced from the subcallosal and orbital fields has been reviewed elsewhere (Kaada 1951, pp. 151—156).

Thus the subcortical area inducing synchronization and behavioural sleep may be influenced not only by *ascending* impulses (*cf* Introduction) but also by *descending* impulses from the cortical level Such an organization would be parallel to the ascending and descending influences on the brain stem desynchronizing or arousal mechanisms (Segundo *et al* 1955 Fangel and Kaada 1960)

The experiments give further evidence of a differentiation within the perigenual cortex of two functionally different zones a subcallosal sleep producing area which coincides with that exerting strong inhibitory effects on cortically and reflexly induced movements decrease in blood pressure, and inhibition of the motility of the uterus, urinary bladder and gastrointestinal tract (Kaada 1960), and a pre and supracallosal cortex which exerts a facilitatory influence on the same activities and from which electrocortical desynchronization and behavioural arousal could be produced Finally the 2 zones have been differentiated on a behavioural basis selective removal of the subcallosal and septal region results in a defect in passive avoidance behaviour (Kaada Rasmussen and Kveim 1962 McCleary 1961) removal of the anterior supracallosal cortex produces a defect in active avoidance (McCleary 1961)

References

AKERT K and B ANDERSSON Experimenteller Beitrag zur Physiologie des Nucleus caudatus. *Acta physiol scand* 1951 22 281—298

AKERT K W P KOELLA and R HESS JR Sleep produced by electrical stimulation of the thalamus *Amer J Physiol* 1952 *168* 260—267

BONVALLET M P DELL and G HIEBEL Tonus sympathique et activite electrique corticale. *Electroenceph clin Neurophysiol* 1954 6 119—144

CLEMENTE C D and M B STERMAN Cortical synchronization and sleep patterns in acute restrained and chronic behaving cats induced by basal forebrain stimulations. *Electroenceph clin Neurophysiol* 1963 (Suppl 24) 172—187

FANGEL C and B R KAADA Behavior "attention and fear induced by cortical stimulation in the cat *Electroenceph clin Neurophysiol* 1960 *12* 575—588

FAVALE E C LOEB G F ROSSI and G SACCO EEG synchronization and behavioral signs of sleep following low frequency stimulation of the brain stem reticular formation *Arch ital Biol* 1961 *99* 1—22

CEREBTZOFF M A Contribution a la physiologie du corps strie *Arch int Physiol* 1941 *51* 333—352

GJONE R Excitatory and inhibitory bladder responses to stimulation of limbic diencephalic and mesencephalic structures in the cat *Acta physiol scand* 1966 *66* 91—102

HERNANDEZ PEON R and G CHAVEZ IBARRA, Sleep induced by electrical or chemical stimulation of the forebrain *Electroenceph clin Neurophysiol.* 1963 (Suppl. 24) 188—198

HESS W R Das Schlafsyndrom als Folge dienzephaler Reizung *Helv physiol pharmacol Acta* 1944 *1* 305—34

KAADA B R Somato-motor autonomic and electrocorticographic responses to electrical stimulation of rhinencephalic and other structures in primates cat and dog *Acta physiol scand* 1951 *24* Suppl 83 1—285

KAADA B R Cingulate posterior orbital anterior insular and temporal polar cortex Pp 1345—1372 in Field F Magoun H W and Hall V E (eds) *Handbook of Physiology* Williams and Wilkins Co Baltimore 1960 vol 2

KAADA B R and N B JOHANNESSEN Generalized electrocortical activation by cortical stimulation in the cat *Electroenceph clin Neurophysiol* 1960 *12* 567—573

KAADA B R E W RASMUSSEN and O KVEIM Impaired acquisition of passive avoidance behavior by subcallosal septal hypothalamic and insular lesions in rats *J comp physiol. Psychol* 1962 *55* 661—670

KAADA B R K WESTER and E ALNES EEC synchronization and behavioral drowsiness induced by low frequency stimulation of the subcallosal region in the unanesthetized cat. *Electroenceph clin Neurophysiol* 1965 *18* 207

LAURSEN A M Corpus striatum *Acta physiol scand* 1963 *59* Suppl 211 1—106

McCLEARY R A Response specificity in the behavioral effects of limbic system lesions in the cat *J comp physiol Psychol* 1961 *54* 605—613

MORUZZI G and H W MAGOUN Brain stem reticular formation and activation of the EEG *Electroenceph clin Neurophysiol* 1949 *1* 455—473

MURPHY J P and E GELLHORN The influence of hypothalamic stimulation on cortically induced movements and on action potentials of the cortex. *J Neurophysiol* 1945 *8* 341—364

POMPEIANO O and J E SWETT Identification of cutaneous and muscular afferent fibers producing EEG synchronization or arousal in normal cats *Arch ital Biol* 1962 *100* 343—380

SEGUNDO J P R NAQUET and P BUSER Effects of cortical stimulation on the electrical activity in the monkey *J Neurophysiol* 1955 *18* 236—245

SETEKLEIV J Uterine motility of the estrogenized rabbit V Response to brain stimulation. *Acta physiol scand* 1964 *62* 313—32

SHIMAMOTO T and M VERZEANO Relation between caudate and diffusely projecting thalamic nuclei. *J Neurophysiol* 1954 *17* 278—288

STERMAN M and C D CLEMENTE Forebrain inhibitory mechanisms Cortical synchronization induced by basal forebrain stimulation *Exp Neurol* 1962 *6* 91—102

STEVENS J R C KIM and P D McLEAN Stimulation of caudate nucleus. Behavioral effects of chemical and electrical excitation *Arch Neurol (Chic)* 1961 *4* 47—65

STOUTEL N and C TERZUOLO Étude des connexions et de la physiologie du noyau caudé *Arch neurol psychiat Belg* 1954 *54* 239—48

URSIN H and B R KAADA Functional localization within the amygdaloid complex in the cat. *Electroenceph clin Neurophysiol* 1960 *12* 1—20

Acta physiol scand 1973 87 103—112

From the Department of Physiology University of Goteborg Sweden

Reflex Bradycardia Elicited from Left Ventricular Receptors during Acute Severe Hypoxia in Cats

By

PETER THOREN

Received 24 April 1972

Abstract

THOREN P *Reflex bradycardia elicited from left ventricular receptors during acute severe hypoxia in cats* Acta physiol scand 1973 87 103—112

In chloralose anesthetized cats blood pressure heart rate and left ventricular pressure were measured as well as changes in ventricular volume by means of a cardiometer Hypoxia was induced by ventilating the animal with a gas mixture containing 3 % O_2 1—2 min after the onset of hypoxia cardiac volume started to increase and simultaneously a bradycardia developed The bradycardia was eliminated by vagal cooling indicating that it was elicited by nervous mechanisms rather than by local myocardial factors After injection of procaine (0 3—0 5 %) into the cardiometer the afferent vagal fibres from the ventricles could be blocked leaving the efferent fibres to the sinus node intact Such selective denervation of the ventricular receptors markedly reduced the bradycardia during hypoxia suggesting that it is mainly a reflex response emanating from ventricular receptors

In a recent publication left ventricular receptors signalling in nonmyelinated vagal afferents and also in other respects closely resembling those reported by Jarisch and Zotterman in 1948 were analysed (Öberg and Thoren 1972 b) These receptors which could elicit a marked reflex bradycardia were activated by *e g* distension of the left ventricle but also during asphyxia or during coronary artery occlusion (Thoren 1972 a) However also in the latter situations the ischemic ventricular distension appeared to be direct cause of the receptor excitation since they started to fire first after a considerable latency and then in close parallel to the appearance of ventricular dilatation

Since bradycardia is a common finding during severe hypoxia and asphyxia it seemed to be of interest to analyse to what extent the mentioned ventricular mechano receptors contribute to this response Skolasinska Sandor and Kovach 1971) have recently suggested that the slowing of the heart during severe anoxia is due to activation of cardiac receptors

A preliminary report of this study has been published earlier (Thoren 1972 b)

Methods

21 cats were used anesthetized with chloralose 30—50 mg/kg after induction with ether. A tracheal cannula was inserted after which the cervical vagal nerves were dissected free and separated from the sympathetic trunks. Both aortic nerves were cut close to their junction with the superior laryngeal nerve. The cervical vagal nerves were placed on cooling devices, which could be cooled to 0 °C by perfusion with an ice-alcohol mixture. The intact right vagal nerve was further placed on a stimulation electrode.

The animals were then placed on artificial respiration with a pump frequency of 19/min and a tidal volume of 15—?0 ml/kg b.w. The thorax was opened through an intercostal incision in the right 6th interspace prolonged over the midline by division of the sternum. The pericardium was opened widely and a snare was placed around the ascending aorta. For recordings of changes in cardiac volume the heart was placed in a cardiometer cup which was made as airtight as possible by means of a rubber membrane embracing the heart smoothly around the atrioventricular groove.

After opening of the thorax 15 – ?0 ml of dextran was injected intravenously. This expansion of the blood volume was supposed to compensate at least partly for the shrinkage of the heart in connection with thoracotomy (*cf.* Rushmer *et al.* 1954). Arterial blood pressure was measured in the brachial artery and recorded on a Grass Polygraph 7 A recorder by means of a Statham P 23 AC pressure transducer. The left ventricular pressure was similarly measured from a catheter PE ?0 advanced into the ventricular cavity from one carotid artery. Heart rate was measured by a tachograph that was triggered by the rapid systolic upstroke of the ventricular pressure. The cardiometer was connected via a 2 l container to a Grass PT 5 volume transducer recording on the Grass polygraph. The femoral artery was cannulated for sampling of blood and determinations of arterial PO_2, PCO_2 and PH (Radiometer Copenhagen type 27).

Experimental procedures. During control conditions the animals were ventilated with a gas mixture containing 96 % O_2 and 4 % CO_2. Hypoxia was induced either by switching off the respirator (3 expts.) or by ventilating the animal with a gas mixture containing 3 % O_2, 4 % CO_2 and 93 % N_2 (13 expts.) or 3 % O_2, 10 % CO_2 and 87 % N_2 (5 expts.). The low oxygen ventilation or the respiratory standstill was maintained for 60—160 s and the animals were allowed to recover for at least 8 min before the next hypoxic period. Initially ?—3 control hypoxic periods were induced. Such periods were then repeated both after cooling and subsequent rewarming of the cervical vagal nerves. Similar hypoxic tests were also carried out after selective blockade of the afferent fibres from cardiac receptors induced by means of injections of procaine HCl (?—5 ml 0.3—0.5 % in saline) into the cardiometer while both atria were continuously rinsed with large amounts of body warm saline in order to avoid a concomitant blockade of the efferent vagal fibres to the sinus node. The presence of an intact efferent innervation was repeatedly tested by efferent electrical stimulation of the right vagus nerve. The blockade of the afferent fibres was considered satisfactory when no bradycardia was observed when the ascending aorta was occluded, a procedure known to cause an intense activation of the receptors (Öberg and Thorén 1972 b). The afferent blockade was interrupted by drainage of the procain solution and rinsing of the epicardial surface with body warm saline.

Results

1) Intact cardiac innervation

Typical responses to acute hypoxia are demonstrated in Fig. 1 left panel. After some latency a marked bradycardia develops. This bradycardia is preceded by an increase in ventricular volume, a rise in left ventricular diastolic pressure and a fall of arterial pressure, suggesting a progressive ischemic dilatation and failure of the ventricles. On return to ventilation with 96 % O_2 and 4 % CO_2 ventricular volume is rapidly normalized as is heart rate.—The typical bradycardia arising from an occlusion of the ascending aorta is also demonstrated in Fig. 1.

A similar pattern of response to hypoxia as demonstrated in Fig. 1 was obtained in all experiments in this series. Thus marked slowing of the heart never occurred

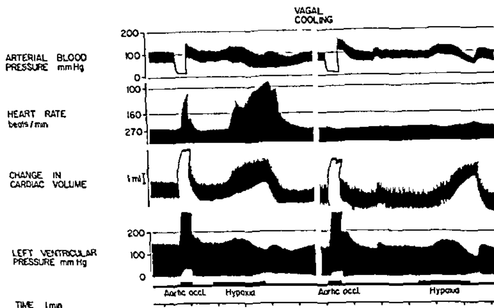


Fig 1 The changes in arterial blood presure heart rate cardiac volume and left ventricular pressure during aortic occlusion and low oxygen breathing (3 % O 4 % CO_2 and 93 % N_2) before and after bilateral cervical vagal cooling

without preceding signs of a substantial ventricular distension In a few cases there occurred a slight and slowly developing dilatation of the ventricles already in the earlier stages of the hypoxic period and which was not accompanied by slowing of the heart Also in these cases however the ventricular volume suddenly started to increase rapidly at a certain point during the hypoxia and this rapid distension was followed by a marked bradycardia

Quantitative data from all the experiments are presented in Table I The extent of the heart rate responses were essentially the same whether the cat was ventilated with 3 % O and 4 % CO in N or with 3 % O and 10 % CO in N but the onset of bradycardia appeared earlier when the gas mixture containing 10 % CO was employed The heart rate responses to an aortic occlusion were quantitatively similar to those obtained during acute hypoxia

To analyse whether the opening of the pericardium might have allowed for an especially marked ventricular dilatation the heart rate responses to hypoxia were compared before and after opening of the pericardium in 4 animals The decrease in heart rate during hypoxia amounted to 62 beats/min (±7 7 S E) when the pericardium was intact as compared to 57 beats/min (±9 5 S E) after opening of the pericardium The heart rate responses to hypoxia were thus not significantly affected by opening of the pericardium

In 7 cats arterial PO and PCO were measured at the onset of the bradycardia during ventilation with 3 % O 4 % CO and 93 % N The mean value for PO

TABLE I Latency to onset of bradycardia and the decrease in heart rate during acute hypoxia induced by ventilation with low oxygen gas mixtures, and during aortic occlusion.

	N	Latency to onset of bradycardia (sec ± S.E.)	Decrease in heart rate (beats/min ± S.E.)
1) Hypoxia 3 O 4 CO 93 (12 exp)	58	3 _ 3 5	87 _ 4
3 O 10 CO, 87 N (4 exp)	10	26 _ 3 1	83 _ 3 9
2) Aortic occlusion	63	(about 1–2 sec)	[illegible] _ 3 4

TABLE II The effect of blocking the cervical vagal nerves by cooling (A) and of blocking the afferent fibres from the left ventricle by injecting procaine into the cardiometer (B) on heart rate changes during aortic occlusion and during acute hypoxia induced by ventilation with 3 O and 4 or 10 CO

Vagal cooling (exp)

A	N	Before cooling	During cooling	p (Students t-test)
Decrease in heart rate during aortic occlusion (beats/min S.E.)	6	63 10 1	−13 _ 4 8 (tachycardia)	0 001
Decrease in heart rate during hypoxia (beats/min S.E.)	7	83 3 9	18 _ 5 7	0 001

Procaine in the cardiometer (9 exp)

B	N	Before procaine	During procaine	
Decrease in heart rate during vagal efferent stim (beats/min S.E.)	19	117 5 6	105 4 6	0 1
Decrease in heart rate during aortic occlusion (beats/min S.E.)	17	87 7 7	8 3 4	0 001
Decrease in heart rate during hypoxia (beats/min S.E.)	18	91 7 0	38 7 6	0 001

was 26 mm Hg (± 3 6 S E) PCO was within the normal range both before and during the hypoxic periods

2) *The responses to hypoxia after vagal cooling*

In 7 cats the heart rate changes during acute hypoxia was tested before and during cervical vagal cooling Fig 1 illustrates that the slowing of the heart during hypoxia is drastically reduced by the vagal blockade Furthermore the response develops with a longer latency —Similarly the bradycardia following aortic occlusion is abolished by vagal cooling and rather reversed to a tachycardia After vagal rewarming the

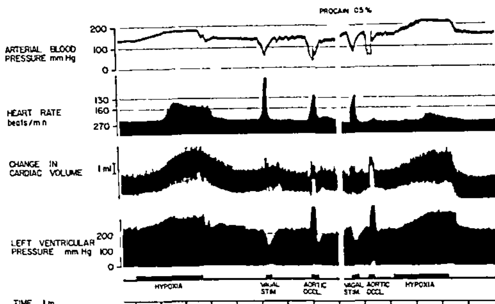


Fig 2 The effects of aortic occlusion vagal efferent stimulation (4 Hz 2 ms 6 V) and hypoxia (3 % O 10 % CO_2 and 87 % N_2) on arterial blood pressure heart rate cardiac volume and left ventricular pressure before and after 0 5 % procaine was injected into the cardiometer

bradycardia response to hypoxia and aortic occlusion re appears Results from these experiments are summarized in Table II A These results thus indicate that an intact vagal innervation of the heart is necessary for the emergence of a bradycardia during the presently used hypoxic periods and that this response is not due to local myocardial factors

However in 2 cats a marked bradycardia could be elicited even after vagal blockade but only when the hypoxic period was markedly prolonged Therefore local myocardial factors may play a role for the heart rate response in hypoxia if the nutritional supply to the myocardium is severely restricted for a long time Data from these two experiments are not included in Table II A

3) The responses to hypoxia after procaine administration into the cardometer

In 13 expts procaine (3—5 ml of a 0 3—0 5 % solution in saline) was injected into the cardiometer so that the epicardial surface of the ventricles became anesthetized With this procedure the afferent fibres from the left ventricular receptors were blocked as indicated by the absence of a bradycardia response to aortic occlusion In 9 of the expts the efferent vagal innervation of the sinus node remained largely intact as shown by the marked bradycardia obtained by efferent vagal stimulation Results from these experiments are summarized in Table II B In the remaining 4 expts procaine blocked also the efferent innervation of the sinus node and data from these experiments are therefore not included in Table II B

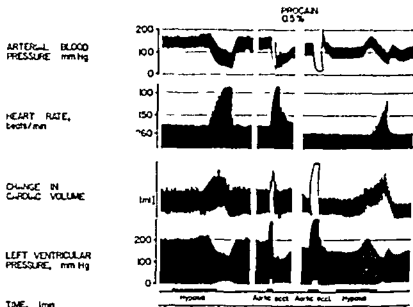


Fig. 3. Changes in arterial blood pressure, heart rate, cardiac volume and left ventricular pressure during aortic occlusion and hypoxia (3 % O_2, 4 % CO_2 and 93 % N_2) early in the experiment (left panel) and 1 1/2 h later (right panel) when the cat is in a bad general condition with lowered blood pressure. (The vagal efferent stimulations are of the same magnitude before and after procaine.) After cervical vagotomy later in the experiment the bradycardia appears with much longer latency.

Fig. 2 shows records from one experiment in this series. The left panel demonstrates the heart rate responses to hypoxia, aortic occlusion and to efferent vagal stimulation before procaine administration in the cardiometer. All these manoeuvres produced, as expected, a marked bradycardia developing in parallel with the ventricular distension. It should also be noticed that the arterial blood pressure recovers fairly slowly after the release of the aortic occlusion.

In the right panel hypoxia, aortic occlusion and efferent vagal stimulation were repeated after procaine blockade of the afferent fibers from the left ventricle. The absence of heart rate responses to aortic occlusion while efferent vagal stimulation still induces the same marked bradycardia as before procaine administration indicates a selective and essentially complete blockade of the afferent pathways in the reflex. Under such conditions the arterial blood pressure recovers immediately upon release of aortic occlusion. This observation strongly suggests that the reflex bradycardia caused by arortic occlusion emanates from ventricular receptors and not from atrial, pulmonary or venous receptors.

When the animal is now exposed to hypoxia which produces the same extent of cardiac distension as before, only a minor bradycardia is observed, indicating that afferent signals from the heart are of great importance for inducing bradycardia during acute hypoxia. To test whether procaine, absorbed into the circulation from the pericardial cavity, might have affected the reflex bradycardia response to aortic

occlusion and hypoxia e g by affecting central vagal motor neurons procaine (1 ml 0 5 % *solution) was injected i v in one* animal This did not affect the reflex heart rate responses to aortic occlusion and hypoxia

In 2 expts a marked bradycardia was elicited during hypoxia, despite a complete blockade of the ventricular receptors with procaine These divergent results were obtained in cats which were in a rather poor condition after several hours of anesthesia and extensive preparations and with somewhat lowered arterial blood pressures Records from one of these experiments are shown in Fig 3 The typical heart rate responses to hypoxia and aortic occlusion are seen in the 2 left panels The right panel shows that the heart rate responses to aortic occlusion is completely eliminated after procain administration in the cardiometer 90 min later in the course of the experiment while hypoxia is still capable of producing a substantial bradycardia However in contrast to the situation before procaine blockade this bradycardia does not arise simultaneously with the hypoxic ventricular dilatation but considerably later Cervical vagotomy later in the experiment markedly reduced the bradycardia during hypoxia indicating that an increased vagal outflow is to a large extent responsible for this response This increased vagal activity must however be initiated from other sources than from the ventricular receptors These experiments are included in Table II B

Discussion

In recent experiments on cats (Thoren 1972 a) it was shown that one group of left *ventricular receptors with non medullated vagal afferents became activated during* asphyxia Since they responded whenever the ventricle was distended *e g* by occlusion of ascending aorta and in connection with asphyxia first when a substantial ventricular dilatation occurred it seems reasonable to suggest that this dilatation *per se* was the direct cause of the receptor activation during asphyxia The receptors also fired with a cardiac rhythm at least periodically which indicates that they function as mechanoreceptors rather than as chemoreceptors Further their activation regularly caused a pronounced reflex bradycardia (Öberg and Thoren 1972 b) Since it is well known from studies on both animals and man (for ref see below) that acute severe hypoxia or a phyxia results in considerable slowing of the heart it was considered of interest to analyse whether and to what extent the mentioned cardiac receptors might contribute to this response It is important to point out that the acute hypoxic periods used in this study lead to such a severe hypoxia that its prolongation beyond a few minutes is fatal No attempts were made to test the effects of gas mixtures with different O contents

Local myocardial factors *per se* were of minor importance at least during a shortlasting severe hypoxia since vagal blockade eliminated or radically reduced the bradycardia response This finding agrees with those reported by Austen *et al* (1963) and by Litwin and Skolasinska (1966) It should be noted however that with prolonged severe hypoxia fairly marked reductions in heart rate were occasionally observed in the present study despite an effective vagal blockade

Therefore with prolonged and extreme hypoxia local myocardial factors may contribute considerably to the bradycardia

Further the present results strongly indicate that the bradycardia during severe hypoxia is a reflex response emanating mainly from ventricular receptors This is evident from the fact that selective blockade of ventricular vagal afferents with procaine into the cardiometer markedly diminished the response It should be emphasized however that a very marked bradycardia was in a few animals seen also after blockade of the ventricular afferents This response must therefore probably be initiated from sources located outside the heart and cerebral asphyxia or hypoxia may then be the cause of these responses (cf Levy Ng and Zieske 1968) — As mentioned these latter results were obtained in deteriorating animals a circumstance which might have contributed to the results in these experiments

Thus 3 different mechanisms may contribute to the bradycardial response to acute hypoxia observed in the present experiments the most important one being a reflex elicited from left ventricular receptors. However an increased vagal efferent discharge may be induced also from extracardial sources and possibly due to cerebral hypoxia or ischaemia Finally also local myocardial factors may in certain circumstances be of importance The mutual importance of these different mechanisms may among other things depend upon the duration and severity of hypoxia the general condition of the animal prior to the hypoxia and to the type of the experimental design

Activation of chemoreceptor afferents are known to elicit bradycardia but only if the ventilation is controlled (Downing Remensnyder and Mitchell 1962 Daly and Scott 1962 Otherwise the increased activity in the pulmonary inflation receptors inhibits the reflex bradycardia or even reverses it to a tachycardia (Angell James and Daly 1969)

The hypoxic bradycardia seen in unanesthetized rabbits has been shown to be due to chemoreceptor activation (Chalmers Korner and White 1967 This decrease in heart rate to acute hypoxia was however markedly diminished if the animals were anesthetized (Korner et al 1968 and was totally abolished by hyperventilation of the anesthetized animal Crocker et al 1968) In the present study there was little indication that chemoreceptor activation was responsible for the bradycardia during the hypoxic tests Thus this response did not develop in parallel to the blood pressure increase obtained in earlier phases of the hypoxia and which was presumably a result of chemoreceptor stimulation Furthermore the pronounced bradycardia studied here was elicited first when PO was reduced below some 30 mm Hg while arterial chemoreceptors are stimulated already when PO_2 is reduced below 70 mm Hg, provided PCO is within a normal range (Hornbein 1968) The apparent absence of a chemoreceptor induced bradycardia may be due to the fact that the animals were placed on artificial respiration and probably somewhat hyperventilated with a tidal volume between 15—20 ml/kg b w It should also be mentioned that the aortic nerves were cut in the present study and the left carotid artery ligated for cannulation purposes and that therefore probably only the chemoreceptors in the

right carotid body may have functioned normally However also the bradycardia seen during acute anoxia in anesthetized dogs was evidently not due to increased activity in chemoreceptor afferents because it was unchanged by chemoreceptor denervation (Litwin and Skolasinska 1966 Skolasinska *et al* 1971)

The present results thus differ from the findings by Göpfert (1947) suggesting that cerebral hypoxia was more prone to induce bradycardia than hypoxia in other parts of the systemic circulation The present findings are also at variance with those of Austen *et al* (1963) who claimed that the bradycardia and cardiac arrest during acute hypoxia was due to an increased vagal tone initiated from receptors located outside the heart They found that cardiac hypoxia (oxygen saturation about 70 %) induced only a slight bradycardia despite intact innervation of the heart while hypoxia in the separately perfused systemic circulation of the same extent induced a marked bradycardia provided the vagal innervation to the heart was intact

Cross *et al* (1963) claim that reflexes from the hypoxic carotid artery territories were the most important factor for producing the severe heart failure with bradycardia seen during acute severe hypoxia in dogs Perfusing both common carotid arteries via an extracorporeal circuit they observed that when lowering the carotid artery PO to 15—45 mm Hg bradycardia and cardiac failure supervened while systemic hypoxia led to bradycardia at PO of 20—25 mm Hg In their isolated heart preparations left ventricular end diastolic pressure began to rise indicating cardiac distension at PO of about 20—30 mm Hg—In contrast Skolasinska *et al* (1971) suggested that cardiac receptors may be of importance for the bradycardia during acute anoxia

In man breathing of low oxygen gas mixture may evoke circulatory collaps with bradycardia (Benzinger Doring Hornberger 1942) This effect was ascribed to a Bezold Jarisch reflex. Later Anderson *et al* (1946) examined the cardiovascular response to hypoxia in man breathing 7—8 % O in N Most of their cases became unconscious without signs of bradycardia but three out of thirteen men fainted suddenly in a typical vasovagal syncope with bradycardia peripheral vasodilation pallor and sweating These latter findings are interesting because the same left ventricular receptors as studied in the present experiments appear to trigger the vasovagal reaction provoked by rapid hemorrhage (Öberg and Thoren 1972 a) After pooling of blood in the legs (about 1 5 l) while being exposed to hypoxia 10 of 13 men fainted with a typical vasovagal reaction (Anderson *et al* 1946) possibly a result of a squeezing of the left ventricle due to the increased contractility (induced by the hypoxia) combined with the reduced venous return A squeezing of the left ventricle of such a type can strongly activate the left ventricular receptors (Öberg and Thoren 1972 a)

In conclusion therefore the bradycardia response to acute severe hypoxia can no doubt be initiated from several different sources but the present results how that the consequent ischemic dilatation of the heart can result in a marked stimulation of ventricular mechano-receptors which in turn produce a profound reflex slowing of the heart

Therefore with prolonged and extreme hypoxia local *myocardial factors* may *contribute considerably to the bradycardia*

Further, the present results strongly indicate that the bradycardia during severe hypoxia is a reflex response emanating mainly from ventricular receptors. This is evident from the fact that selective blockade of ventricular vagal afferents with procaine into the cardiometer markedly diminished the response It should be emphasized however that a very marked bradycardia was in a few animals seen also after blockade of the ventricular afferents. This response must therefore probably be initiated from sources located outside the heart and cerebral anoxia or hypoxia may then be the cause of these responses (*cf* Levy Ng and Zieske 1968) — As mentioned these latter results were obtained in deteriorating animals a circumstance which might have contributed to the results in these experiments

Thus 3 different mechanisms may contribute to the bradycardial response to acute hypoxia observed in the present experiments the most important one being a reflex elicited from left ventricular receptors However an increased vagal efferent discharge may be induced also from extracardial sources and possibly due to cerebral hypoxia or ischaemia Finally also local myocardial factors may in certain circumstances be of importance The mutual importance of these different mechanisms may among other things depend upon the duration and severity of hypoxia the general condition of the animal prior to the hypoxia and to the type of the experimental design

Activation of chemoreceptor afferents are known to elicit bradycardia but only if the ventilation is controlled (Downing Remensnyder and Mitchell 1962 Daly and Scott 1962) Otherwise the increased activity in the pulmonary inflation receptors inhibits the reflex bradycardia or even reverses it to a tachycardia (Angell James and Daly 1969)

The hypoxic bradycardia seen in unanesthetized rabbits has been shown to be due to chemoreceptor activation (Chalmers Korner and White 1967) This decrease in heart rate to acute hypoxia was however markedly diminished if the animals were anesthetized (Korner *et al* 1968) and was totally abolished by hyperventilation of the anesthetized animal (Crocker *et al* 1968) In the present study there was little indication that chemoreceptor activation was responsible for the bradycardia during the hypoxic test Thus this response did not develop in parallel to the blood pressure increase obtained in earlier phases of the hypoxia and which was presumably a result of chemoreceptor stimulation Furthermore the pronounced bradycardia studied here was elicited first when PO_2 was reduced below some 30 mm Hg while arterial chemoreceptors are stimulated already when PO_2 is reduced below 70 mm Hg provided PCO_2 is within a normal range (Hornbein 1968) The apparent absence of a chemoreceptor induced bradycardia may be due to the fact that the animals were placed on artificial respiration and probably somewhat hyperventilated with a tidal volume between 15—20 ml/kg b w It should also be mentioned that the aortic nerves were cut in the present study and the left carotid artery ligated for cannulation purposes and that therefore probably only the chemoreceptors in the

Acta physiol scand 1973 87 113—120

From the Psychiatric Research Center Ulleråker Hospital, University of Uppsala Sweden

Increased N-Acetylserotonin and Melatonin Formation Induced by d Amphetamine in Rat Pineal Gland Organ Culture via a β-Adrenergic Receptor Mechanism

By

MARIA BACKSTROM and LENNART WETTERBERG

Received 28 April 1972

Abstract

BACKSTROM M and L WETTERBERG *Increased N acetylserotonin and melatonin formation induced by d amphetamine in rat pineal gland organ culture via a β adrenergic receptor mechanism* Acta physiol scand 1973 *87* 113—120

Dextro-amphetamine (10^{-5} M) added to the medium of rat pineal glands in organ culture produces an eightfold increase of radiolabeled N acetylserotonin and melatonin when tryptophan or serotonin are used as labeled precursors In concentration of 10^{-4} M d amphetamine causes a decreased formation of 5 hydroxyindoleacetic acid from tryptophan and serotonin Addition of d amphetamine to pineal glands also resulted in an increase of N acetyltransferase activity as compared with untreated glands The effect of d amphetamine and noradrenaline was blocked by propranolol a β adrenergic blocking agent but not by the α adrenergic blocking agent phentolamine In cervical ganglionectomized rats the addition of d amphetamine to the culture medium did not produce an increase in N acetylserotonin production Conversely noradrenaline added to the medium gave a high formation of N acetylserotonin This indicates that the major effects of d amphetamine on pineal N acetyltransferase activity are mediated by noradrenaline

A recently developed method for organ culture of the rat pineal gland provides an experimental model for *in vitro* studies which may help to identify some of the mechanisms responsible for the effect of amphetamine on neuroendocrine tissue

In the present study we report the influence of d amphetamine on serotonin metabolism and N acetyltransferase activity in unoperated control and ganglionectomized rats

We also report that the d amphetamine effect on pineal gland metabolism is mediated by a mechanism which can be blocked by addition of an adrenergic β receptor blocking agent but not by addition of an alpha receptor blocking agent to the organ culture medium

Materials and methods

The pineal glands were cultured according to a technique developed by Trowell (1959) and modified by Rasz (1963) as described in detail by Klein and Weller (1970).

Watch glasses fitted with bent steel meshes were used for culture vessels. Seven vessels were placed on filter paper in a Petri dish. The basic culture medium used was BGJ_b Fitton-Jackson modification (Grand Island Biological Company). Bovine serum albumin (1 mg/ml), ascorbic acid (0.1 mg/ml medium) and 1 glutamine (0.01 mg/ml) were added on the day before the experiment. Tryptophan and serotonin concentrations in the medium were 0.04 mg/ml and 0.1 mg/ml respectively.

The culture vessels were each filled with 0.6 ml of sterile medium containing either L-(^3H) tryptophan (New England Nuclear Comp. Boston, Mass.) or (^{14}C) serotonin (Amersham, Searle Co. England). The filter paper was soaked with 2.5 ml of sterile Eagle medium. Vessels and medium were equilibrated in a gas-tight chamber (37 °C, 95 % O_2, 5 % CO_2) for 1 h before one pineal was put on top of each steel mesh.

Pineal glands were taken from male Sprague-Dawley rats (160–180 g) sacrificed by decapitation. The animals were kept on a 12–12 h light–dark schedule with lights on between 6 a.m. and 6 p.m. Bilateral superior cervical ganglionectomy was performed on the animals 3 weeks prior to the experiment. This group of rats were otherwise kept under the same conditions as the unoperated controls. The pineals were removed between 10 and 12 a.m., immediately dissected free, stored in sterile culture medium on ice and placed in the culture vessels within a few minutes.

The tissue was preincubated for 1 h before drugs were added. The receptor blockers, when used, were added 30 min before addition of the following drugs. After 24 h of incubation radiolabeled N-acetylserotonin, melatonin and 5-hydroxyindoleacetic acid in the media were isolated by thin layer chromatography (Klein and Notides 1969), eluted and the radioactivity measured in a Packard Tri-Carb Liquid Scintillation Spectrometer.

In some experiments pineal glands were removed from the media after 6 h of incubation and homogenized in phosphate buffer. N-acetyltransferase activity in the homogenates was assayed according to Klein, Berg and Weller (1970), incubating the pineal homogenates for 10 min. Statistical analyses were performed by Student's t test.

Other chemicals: Phentolamine (CIBA), l-norepinephrine bitartrate (Sigma), propranolol hydrochloride (ICI), d-amphetamine sulphate (ACO).

Results

Addition of noradrenaline to the nutrient medium of the rat pineal gland organ cultures in concentrations of 10^{-5} and 10^{-6} M caused an increase in the formation of N-acetylserotonin and melatonin from (^3H) tryptophan (Table I) as well as from (^{14}C) serotonin (Table II). It also caused an increased N-acetyltransferase activity (Table III). The production of metabolites from the glands after addition of noradrenaline to the organ culture medium differs significantly from control values (Table I and II). A scheme of the synthesis of N-acetylserotonin and melatonin from tryptophan in the pineal gland is shown in Fig. 1.

In pineal glands from chronically denervated rats the addition of noradrenaline (10^{-6} M in the medium) produced a marked increase in the formation of N-acetylserotonin as compared to control glands from both denervated and intact rats while no stimulation of melatonin production was observed (Table II).

Addition of d-amphetamine in concentrations of 10^{-4}, 10^{-5} and 10^{-6} M to the pineal glands from intact rats caused an increase in the formation of N-acetylserotonin and melatonin from (^3H) tryptophan and (^{14}C) serotonin (Table I and II respectively). Maximal stimulatory effect was achieved by addition of 10^{-5} M d-amphetamine to culture medium which gave 30 per cent more radiolabeled

TABLE I Production of serotonin (5 HT) derivatives by cultured pineal gland from (H) tryptophan

Treatment	Conc in medium (M)		Metabolites in medium		
			N Acetyl 5-HT	Melatonin	5 HIAA
Control		(17)	90±19	373±78	1677±182
Noradrenaline	10^{-}	(11)	563±121**	1422±163 *	1290±132
Noradrenaline	10	(7)	210±87	9 0±186	1910±334*
d Amphetamine	10 *	(9)	492±70**	2446±111**	527±113**
d Amphetamine	10 *	(7)	829±142**	2729±154**	942±93**
d Amphetamine	10^{-4}	(5)	248±28 *	984±230**	1148±278
d Amphetamine	10	(6)	154±83	594±211	766±201*
d Amphetamine + Propranolol	10^{-4} 10^{-}	(4)	62±31	121±22	672±112
d Amphetamine + Phentolamine	10^{-4} 10	(4)	289±48	1933±123	368±44

Data are expressed as mean±S E of picomoles product formed per gland per 24 h incubation period In parenthesis the number of pineal glands per group
5 HIAA = 5 hydroxyindoleacetic acid
* and * designates groups that differ significantly in amounts of metabolites formed from (H) tryptophan from control value * = $P < 0.01$ ** = $P < 0.001$

TABLE II Production of serotonin (5-HT) derivatives by cultured pineal glands from (C) 5 HT

Treatment	Conc in medium (M)		Metabolites in medium		
			N Acetyl 5-HT	Melatonin	5-HIAA
Normal pineals					
Control		(8)	136±38	462±66	4002±552
Noradrenaline	10	(7)	715±104 *	858±48*	3500±379
Noradrenalin	10^{-}	(4)	509±149	862±154	4741±471
d Amphetamine	10	(12)	885±121**	1482±87*	2526±223 *
d Amphetamine	10	(3)	1340±100**	1653±195**	4093±381
Chronically denervated pineals					
Control		(3)	27±7	391±181	3877±581
Noradrenaline	10	(3)	1602±388*	369±53	3694±729
d Amphetamine	10^{-}	(3)	66±12	461±224	3940±862

Data expressed as picomoles product formed per gland per 24 h incubation period In parenthesis the number of pineal glands per group 5 HIAA = 5 hydroxyindoleacetic acid
and ** designates groups that differ significantly in amounts of metabolites formed from (C) 5 HT from control value = $P < 0.01$ * = $P < 0.001$

N acetylserotonin from both precursors than when 10^{-4} M was used The induction of N acetylserotonin and melatonin formation following addition of d amphetamine 10^{-6} M is of a similar magnitude as the one produced when 10^{-5} M noradrenaline is added to the culture medium d Amphetamine in the concentration of 10 M did not show any effect on the serotonin metabolism in the pineal gland The format of 5 hydroxyindoleacetic acid from (^{3}H) tryptophan and (^{14}C) serotonin was

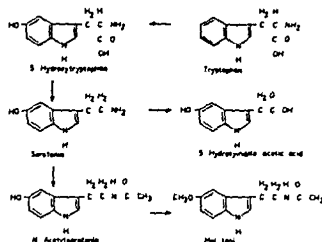


Fig 1 Biosynthesis of N acetyl serotonin melatonin and 5-hydroxyindoleacetic acid from tryptophan in the pineal gland.

cantly decreased ($p < 0.001$) when d amphetamine was added in a concentration of 10⁻⁴ M to the medium whereas d amphetamine 10 M only decreased the formation of 5 hydroxyindoleacetic acid from (^{3}H) tryptophan

Addition of the adrenergic β receptor blocker propranolol (10⁻⁵ M in the medium) totally abolished the effect of both noradrenaline and d amphetamine The α receptor blocker phentolamine (10⁻⁴ M in the medium) did not affect the stimulation following either of the two drugs (Table I) Phentolamine added to the medium as the only substance gave a production of metabolites that did not differ from control values

TABLE III acetyltransferase activity in pineal gland homogenate after 6 h of organ culture with noradrenaline or d amphetamine

Treatment	Conc in media (M)	N	N acetyltransferase activity picomoles product formed per gland per hour
Control		(12)	148 ± 29
Noradrenaline	10	(5)	11 0 ± 160*
Noradrenaline	10	(11)	41[illegible] ± 69
d Amphetamine	10	(5)	1 88 ± 9[illegible]
d Amphetamine	10	(7)	2431 ± 231*
d Amphetamine	10⁻⁴	(6)	1?48 ± 362
d Amphetamine	10	(6)	119 ± ?7

The results are expressed as mean ± S E
* designates groups that differ significantly in enzyme activity from control value $P < 0.001$
N = number of pineal glands assayed

The N acetyltransferase activity measured in pineal gland homogenate was increased after addition of d amphetamine (10^{-4}, 10^{-5} and 10^{-6} M in the medium) to pineal glands in organ culture for 6 h (Table III) The N acetyltransferase activity following addition of noradrenaline or d amphetamine is three to sixteenfold above control values (Table III) The stimulation of enzyme activity induced by d amphetamine (10^{-4} M) is twice as effective as compared to the stimulatory effect of noradrenaline (10^{-4} M)

When d amphetamine (10^{-4} M) was added to the culture medium of glands from sympathectomized rats the formation of N acetylserotonin melatonin and 5 hydroxyindoleacetic acid from (^{14}C) serotonin did not differ from values obtained from denervated control glands (Table II) However when d amphetamine was added to a gland obtained from a fully innervated rat the amount of N acetylserotonin was about 20 times higher and the melatonin approximately four times higher than the production of the same metabolites in a denervated gland (Table III) The 5 hydroxyindoleacetic acid remained at a constant level

Discussion

The present observations confirm that noradrenaline increases the formation of (^{3}H) or (^{14}C) N acetylserotonin and (^{3}H) or (^{14}C) melatonin from labeled tryptophan or serotonin (Axelrod Shein and Wurtman 1969 Wurtman *et al* 1969 Klein and Weller 1970 Shein 1971) and that noradrenaline stimulates the activity of the N acetyltransferase enzyme in rat pineal glands in organ culture (Klein Berg and Weller 1970) These effects of noradrenaline have been shown to be inhibited by the addition of the β adrenergic receptor blocking drug propranolol to the pineal gland organ culture (Wurtman Shein and Laren 1971) The stimulatory effect of noradrenaline is unchanged after addition of the α adrenergic receptor blocking drug phenoxybenzamine (Wurtman *et al* 1971) The present study shows that another α adrenergic blocking drug phentolamine does not affect the increased formation of N acetylserotonin and melatonin following noradrenaline addition We have also confirmed the finding of the blocking effect of propranolol

The data presented show that the treatment of rat pineal glands in organ culture with d amphetamine gives a markedly increased production of N acetylserotonin and melatonin from (^{3}H) tryptophan This increase even exceeds the stimulation achieved after noradrenaline treatment Noradrenaline is probably metabolized under the conditions present during organ culture Thus the effect of noradrenaline on the pineal gland would be transient and it seems likely that d amphetamine is more stable and can exert its effect during a longer period of time The 5 hydroxyindoleacetic acid levels are significantly decreased in medium containing 10^{-4} M d amphetamine when (^{3}H) tryptophan is used as the precursor isotope This finding is well in accordance with earlier reports that d amphetamine in high concentrations causes an inhibition of monoamine oxidase activity (Blaschko Richter and Schlossman 1937 Glowinski Axelrod and Iversen 1966)

Addition of receptor blocking drugs to the organ culture with d-amphetamine shows a similar pattern as when the blockers are combined with noradrenaline. Thus the α-receptor blocking drug phentolamine does not alter the d-amphetamine-induced stimulation of N-acetylserotonin or melatonin formation from (^{14}C) serotonin. The β-receptor blocking agent propranolol gives a total extinction of the d-amphetamine effect. This shows that the effect of d-amphetamine is probably mediated through the same type of receptor as the noradrenaline effect. This could either be due to a direct stimulation of d-amphetamine on the receptor mechanism or an increased release or diminished reuptake of the noradrenaline present in the pineal gland following d-amphetamine treatment. In order to decrease the content of endogenous noradrenaline in the pineal glands we performed bilateral cervical ganglionectomy on a group of male rats. This procedure causes a degeneration of all sympathetic nerves to the rat pineal gland (Owman 1965). The ganglionectomized rats were otherwise kept under the same conditions as the unoperated animals.

Addition of d-amphetamine to the culture medium of a denervated gland gives less than [illegible] per cent of the N-acetylserotonin formed from an innervated gland treated the same way. This clearly shows that the effect of d-amphetamine on the rat pineal gland receptor requires the presence of noradrenaline and thus probably acts indirectly via release of noradrenaline from noradrenergic nerve terminals in the pineal gland.

We also cultured pineal glands with (^{14}C) serotonin as the precursor to investigate whether the increased formation of labeled N-acetylserotonin and melatonin could be caused by an increased uptake of tryptophan into the gland. The increase in formation of metabolites followed the same pattern after both noradrenaline and d-amphetamine treatment as when tryptophan was used. The stimulatory effect therefore is not due to a change in tryptophan uptake. When (^{14}C) serotonin was used as precursor no decrease in the production of 5-hydroxyindoleacetic acid released into the medium can be seen after the addition of d-amphetamine in concentration 10^{-5} M and only a 30 per cent decrease in concentration 10^{-4} M. One possible explanation could be that serotonin is metabolized by the deaminating enzyme during the 1 h long pre-incubation period before the d-amphetamine is added. To form serotonin (^{3}H) tryptophan has to be converted by two enzymatic steps involving hydroxylation and decarboxylation. This causes a time lag and d-amphetamine has already exerted its inhibitory effect on the monoamine oxidase activity before significant amounts of serotonin are available for further conversion.

In accordance with previous findings (Klein *et al.* 1970) the increased formation of N-acetylserotonin and melatonin following treatment with noradrenaline in the rat pineal gland organ culture is paralleled and consequently satisfactorily explained by an increased N-acetyltransferase activity. Likely the stimulation after addition of d-amphetamine corresponds to a striking increase of the N-acetyltransferase activity.

d-Amphetamine 10 [illegible] M causes about twice as high activity of N-acetyltransferase as noradrenaline 10 [illegible] M in pineal glands treated with either drug in the medium for

6 h in organ culture d Amphetamine in a concentration of 10^{-7} M lacked stimulatory effect in the present *in vitro* system

Noradrenaline addition to chronically denervated rat pineal glands in organ culture produces three times as much N acetylserotonin as compared to a gland from an intact rat after the same treatment This could be explained by the increased sensitivity to noradrenaline in sympathectomized tissues connected with a more marked increase in adenyl cyclase activities (Weiss and Costa 1967) and possibly an increased stimulation of N acetyltransferase activity (Klein, Weller and Moore 1971)

N acetyltransferase has been proposed to be activated by adenyl cyclase (Klein and Weller 1970) Noradrenaline increases adenyl cyclase in pineal homogenate but d ampethamine failed to do so (Weiss and Costa 1968) This suggests that d amphetamine increases enzyme activity in intact tissue but not in broken cell preparations

The 50 per cent decrease in melatonin production in noradrenaline treated denervated glands probably follows the decrease in hydroxyindole O methyl transferase (HIOMT) activity which has been reported to occur after sympathectomy (Wurtman Axelrod and Fischer 1964) The substrate dependent increase in melatonin production from N acetylserotonin is to be taken into consideration only when the HIOMT activity exceeds a certain level and is not rate limiting

The 5 hydroxyindoleacetic acid production is unaffected by denervation The monoamine oxidase activity is thus not affected by the disturbance in adrenergic innervation to a degree that alters the flow of serotonin through this metabolic pathway

The observations that d amphetamine induces an enzyme shunting the metabolism of serotonin from 5 hydroxyindoleacetic acid to N acetylserotonin by stimulating a β adrenergic receptor might be of some importance in the understanding of the effects caused by d amphetamine

The authors are highly indebted and gratfeul to Dr D C Klein for his help starting the pineal organ culture system in our laboratory We thank Agnetha Johansson and Siv Beijer for kilful technical assistance This study was supported by grants from Swedish Medical Research Council B 72 21 X 3371 02 B and Anton och Dorotea Bexelius minnesfond

References

AXELROD J H M SHEIN and R J WURTMAN Stimulation of C^{14} melatonin synthesis from C^{14} tryptophan by noradrenaline in rat pineal in organ culture *P oc nat Acad Sci* (Wash) 1969 *6* 544—549

BLASCHKO H D RICHTER and H SCHLOSSMAN The oxidation of adrenaline and other amines *Biochem J* 1937 *31* 2187—2196

GLOWINSKI J J AXELROD and L L IVERSEN Regional studies of catecholam n s in the rat brain IV Effect of drugs on the disposition and metabolism of ^{3}H norepinephrine and ^{3}H dopamine *J Pharmacol exp Therap* 1966 *153* 30—41

KLEIN D C and A NOTIDES Thin layer chromatographic separat on of p ne l gland d r a ti es of serotonin ^{14}C *Analyt B ochem* 1969 *31* 480—483

KLEIN D C and J WELLE Input and output signals in a modeneural s stem the regulation of melatonin produ tion in the pineal gland *In Vitro* 1970 6 197—204

KLEIN D C G R BERG and J WELLER Melatonin synthes s Adeno ne 3 5 monophosphate and norepinephrine st mulate N acetyltransferase *S ience* 1970 *168* 979—980

KLEIN D C, J WELLER and R Y MOORE Melatonin metabolism neural regulation of pineal serotonin acetyl coenzyme A N acetyltransferase activity *Proc nat Acad Sci* (Wash) 1971 *68* 3107—3110

OWMAN C H Localization of neuronal and parenchymal monoamines under normal and experimental conditions in the mammalian pineal gland *Progr Brain Res* 1965 *10* 4 3—153

RAISZ L G Bone resorption in tissue culture Factors influencing the response to parathyroid hormone *J clin Invest* 1965 *44* 103—116

SHEIN H M Control of melatonin synthesis by noradrenaline in rat pineal organ culture *In Ciba Foundation Symposium on the Pineal Gland* (Edit by Wolstenholme G) Churchill, London 1971 197—207

TROWELL O A The culture of mature organs in a synthetic medium *Exp Cell Res* 1959 *16* 118—147

WEISS B and E COSTA Adenyl cyclase activity in rat pineal gland Effects of chronic denervation and norepinephrine *Science* 1967 *156* 1750—1752

WEISS B and E COSTA Selective stimulation of adenyl cyclase of rat pineal gland by pharmacologically active catecholamines *J Pharmacol exp Ther* 1968 *161* 310—319

WURTMAN R J, J AXELROD and J E FISCHER Melatonin synthesis in the pineal gland Effect of light mediated by the sympathetic nervous system *Science* 1964 *143* 1328—1330

WURTMAN R J, H M SHEIN and F LARIN Mediation by β adrenergic receptors of effect of norepinephrine on pineal synthesis of (^{14}C)serotonin and (^{14}C)melatonin *J Neurochem* 1971 *18* 1683—1687

WURTMAN R J, H M SHEIN, J AXELROD and F LARIN Incorporation of ^{14}C tryptophan into ^{14}C protein by cultured rat pineals stimulation by l norepinephrine *Proc nat Acad Sci* (Wash) 1969 *62* 749—755

Acta physiol scand 1973 87 121—132

From the Department of Physiology University of Goteborg Sweden

Circulatory Responses to Stimulation of Medullated and Non medullated Afferents in the Cardiac Nerve in the Cat

By

BENGT ÖBERG and PETER THORÉN

Received 4 May 1972

Abstract

ÖBERG B and P THORÉN *Circulatory responses to stimulation of medullated and non medullated afferents in the cardiac nerve in the cat* Acta physiol scand 1973 87 121—132

Recordings of the evoked compound potentials in the right main cardiac nerve in the cat demonstrated the presence of both medullated and non medullated fibres The circulatory responses to afferent stimulation of these two fibre populations were followed in terms of changes in blood pressure heart rate and skeletal muscle and renal flow resistances A selective stimulation of the medullated afferents which probably arise mainly from atrial receptors induced a moderate reflex rise of blood pressure an increased heart rate and a vasoconstriction in the skeletal muscle and kidney Clearcut effects were observed first with high stimulation frequencies and then only in case the buffering influences from other receptor sites were minimized Stimulation of the non medullated fibres which probably emanate from ventricular receptors caused a very marked reflex blood pressure fall slowing of the heart and vasodilatation in the muscle and the kidney already with very low stimulation rates These results suggest that the powerful reflex influence from cardiac receptors on the cardiovascular system is exerted by receptors with non medullated afferents while endings firing in medullated pathways as *e g* atrial receptors have very small direct effects on the cardiovascular system and then rather of an excitatory nature

Cardiac receptors have generally been assumed to exert a generalized inhibitory influence on the bulbar cardiovascular centre This assumption is based on the findings of a blood pressure fall a slowing of the heart and a vasodilatation when afferent fibres from the heart are electrically stimulated (Jarisch and Zotterman 1948 Neil and Zotterman 1950 Jones 1953 Öberg and White 1970) and when cardiac receptors are chemically excited by *e g* veratrum alkaloides (*cf* Dawes and Comroe 1954) or by a more natural type of stimulus as *e g* pressure elevations in isolated heart chambers (Aviado and Schmidt 1955 Salisbury Cross and Rieben 1960)

A preliminary report on this study has been published earlier (Öberg and Thorén 1971)

However, the cardiac receptors constitute by no means a homogenous group of nerve endings. They differ not only with respect to their location in the atria and in the ventricles and in deep and superficial layers of the myocardium but also with regard to the type of afferent nerve connection. Thus atrial receptors of the classical type morphologically described by Nonidez (1937) as well as some ventricular receptors (e.g. Paintal 1963), are known to signal in medullated fibres while a large population of ventricular receptors seem to fire via non medullated afferents (Jarisch and Zotterman 1948, Sleight and Widdicombe 1965, Öberg and Thorén 1972). It seems likely that such dissimilarities with regard to receptor location and type of afferent pathway are correlated to quite different functions of the separate receptor groups.

Recent studies have shown that stimulation of left atrial receptors located in pulmonary vein atrial junction leads to a reflex increase in heart rate (Ledsome and Linden 1964). This implies that contrary to earlier beliefs some cardiac receptors exert an excitatory influence on the medullary cardiovascular centre at least on the central cardiac neurons.

The present study was undertaken in order to analyse the circulatory responses to an increased activity in medullated and non medullated cardiac afferents respectively. For this purpose the right cardiac nerve was stimulated electrically in afferent direction with such stimulation characteristics that on one hand the medullated afferent fibres were selectively excited and on the other with higher stimulation intensities so that also the non medullated afferent fibres were excited.

Methods

Experiments were performed on 34 cats anesthetized with chloralose (30–[illegible] mg/kg b.w.). A tracheal tube was inserted and the animal placed on a positive pressure artificial respiration and ventilated with [illegible] % O_2 and 4 % CO_2 in most experiments. The chest was opened by means of a right intercostal incision in the 5th interspace. The azygos vein was divided and the right cardiac nerve, running from the heart to the vagal stem in the chest, was freed, cut distally and placed on an electrode for electrical stimulation. In most cats there was only one main cardiac nerve but in some cats two parallel branches were at hand and in these cases both branches were mounted on the electrode. The anatomy in this region has been described in detail (Andrew 1968, ... sympathetic branch ... (Fig. 2)).

Stimuli were delivered from a Grass type S4 stimulator. Arterial blood pressure was recorded from a femoral artery via a Statham P 23 AC transducer. Heart rate was followed by means of a tachograph triggered by the rapid systolic rise of the pressure curve. In 28 cats calf muscle blood flow was measured by cannulating the right popliteal vein in peripheral direction and diverting the venous outflow through a drop chamber operating and ordinate recorder unit. The ordinate recorder pen usually climbed with a constant speed, in which case the heights of the ordinate became in principle inversely proportional to blood flow. In a few experiments, however, the raising speed of the pen was made to vary in proportion to the prevailing blood pressure level by means of an electronic device. The height of the ordinates will in these cases be directly proportional to the flow resistance in the calf muscles.—In 19 expts renal blood flow was similarly recorded by cannulation of the left renal vein and diverting the venous outflow through a drop recorder.—Recordings of the circulatory parameters were made on a Grass Polygraph recorder.

The pH of arterial blood samples was—in most experiments—measured at regular intervals by a Radiometer type [illegible] pH meter and deviations from normal were corrected by iv injections of sodium bicarbonate (1—3 ml 1 M $NaHCO_3$).

The carotid arteries, the vago-sympathetic trunks and the aortic nerves were dissected free bilaterally in the neck. The aortic nerves were cut bilaterally and the central end of one of

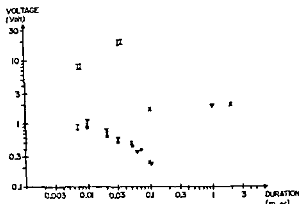


Fig 1 Stimulation strength—duration curves for the different fibre groups in the cardiac nerve The first clear evidence of a compound potential on the oscilloscope was considered as the stimulation threshold Data are collected from 3 expts each one represented by one symbol In 2 expts (crosses and trinagles) 3 clearcut fibre groups were found while in the third experiment (filled circles) only 2 groups were found

them placed on an electrode for stimulation purposes —Bleeding was made from one cannulated femoral or brachial artery Cold block of the right cervical vagal nerve were performed in a few expts by packing frozen saline around the nerve In four experiments the compound action potentials evoked by stimulation of the cardiac nerve were recorded from the same nerve 5—8 mm proximal from the stimulation site by means of a bipolar recording electrode connected to a double beam oscilloscope Tektronix 502 via a differential preamplifier The oscilloscope tracings were photographed The sweep of the oscilloscope was triggered by the stimulation From such recordings the conduction velocities of the different fibre groups could be estimated and the fibres therefore classified as medullated and non medullated fibres Moreover the stimulation thresholds for the different fibre groups could be determined

Results

2 *Stimulation threshold and conduction velocities*

In an initial series of three experiments the stimulation thresholds for the different fibre groups in the right main cardiac nerve were estimated by varying the stimulation intensity and duration independently and recording the evokel compound potentials proximally on the same nerve The lowest intensity and shortest stimulation duration respectively required to produce a clearcut potential on the oscilloscope was taken as the threshold value The maximal compound potential was of course obtained first with higher stimulation intensities Data from the three experiments are shown in Fig 1 In two experiments the cardiac nerve contained 3 clearly separate fibre populations indicated as I II and III respectively In the third experiment only the groups I and III were found

Conduction velocities in the various fibre groups were estimated in 4 expts from the time lag between the stimulation artefact and the compound potential and the distance between the stimulating and recording electrodes The mean conduction velocities for the fastest and slowest conducting fibres in the three groups were for group I 11 and 30 m/s resp for group II (found only in 3 animals) 6 and 11 m/s resp and for group III 0 4 and 1 1 m/s resp It should be realized however that exact figures for conduction velocities are difficult to establish with the present experimental setup because of the relatively short distance between stimulation and recording electrodes It seems clear however that the groups I and II represent medullated fibres and group III non medullated fibres

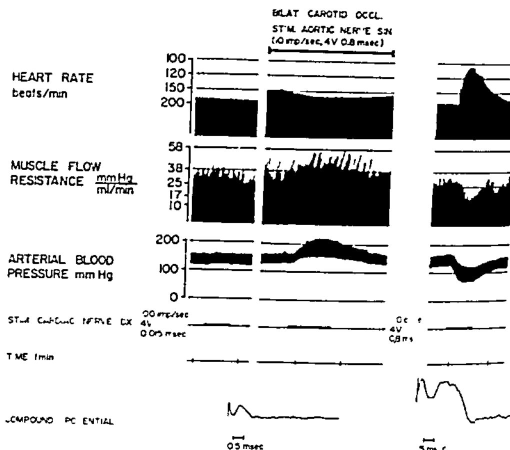


Fig ? Effects of stimulation of medullated (left and middle panel) and non medullated fibres right panel in the main right cardiac nerve on blood pressure heart rate and flow ... ce in calf muscle The evoked potentials recorded proximall from the same nerve are also shown Note the augmented responses to stimulation of the medullated fibres when the buffering influence from arterial baroreceptors are largely removed (carotid occlusion) and a ... ant inhibit r restraint is placed on the vasomotor centre by stimulation of the aortic nerve middle panel)

2 *Circulatory responses to stimulation of the myelinated fibre groups*

From the data presented in Fig 1 it was possible to choose such stimulation characteristics that the low threshold medullated fibre group was electively and maximally stimulated Usually 4 V 0 01—0 02 ms and 60—80 imp/s were chosen as stimulation characteristics

If the stimulation was applied when the carotid arteries were unclamped and the carotid sinus baroreceptors thus allowed to display their full buffering influence no or only very small circulatory responses were induced They consisted of slight increases of blood pressure heart rate and calf muscle and renal flow resistances Records from one experiment are illustrated in Fig 2 left panel and the results of all ...ulat ons in this series are summarized in Table I A

TABLE I Changes in blood pressure heart rate muscle and renal PRU produced by stimulation of the right cardiac nerve (60–80 imp/s 0 01–0 05 ms 4 V) when the carotid sinus baroreceptors remained intact (A) and when the carotid arteries were occluded and a continuous inhibition was placed on the medullary cardiovascular centres by a constant afferent stimulation of one aortic nerve (B)
n = number of stimulations

		Blood pressure change (Range of variation)	Heart rate change (Range of variation)	Muscle PRU change (Range of variation)	Renal PRU change (Range of variation)
A	Intact carotid baroreceptors (33 cats)	4 (0–27) n = 43	2 (0–11) n = 43	8 (0–46) n = 38	3 (0–14) n = 20
B	Bilat carotid occl + Stimulation of one aortic nerve (24 cats)	18 (0–43) n = 47	8 (0–38) n = 47	19 (0–55) n = 47	9 (−7–55) n = 32

The circulatory responses to low intensity stimulation of the cardiac nerve were found to become considerably augmented if the buffering influences from the carotid sinus baroreceptors were minimized by clamping both carotid arteries and if the bulbar cardiovascular centres were simultaneously exposed to a constant inhibitory influence from *e g* afferent stimulation of one aortic nerve In such cases clearcut blood pressure elevations and increases of heart rate and muscle and renal flow resistances were usually obtained although the magnitude of the responses were on the whole still fairly small This is shown in the middle panel of Fig 2 and in Table I B which summarizes the results of all stimulations performed while the carotid arteries were clamped and one aortic nerve stimulated These findings thus suggest that an increased activity in the medullated afferents in the cardiac nerve leads to only moderate circulatory responses of an excitatory nature and that clearcut effects are produced only in case the buffering influences from other receptor sites are eliminated and the prevailing vasomotor centre activity is at the same time maintained low

In a separate series of 5 expts the blood pressure was maintained constant during the stimulation periods by means of a slow withdrawal of blood from one brachial artery In this way pressure dependent autoregulatory alterations of flow resistances in the two studied circuits were avoided The results from these experiments did not differ significantly from those obtained in animals where the blood pressure was allowed to vary during the stimulation period

In contrast to the present observation that a renal vasoconstriction usually occurred when the medullated fibres in the cardiac nerve were stimulated Karim *et al* (1971) have recently reported a reduced activity in efferent sympathetic fibres to the kidney when atrial receptors known to fire in medullated afferents were stimulated To exclude the possibility that the absence of a reflex renal vasodilatation in the present study was simply due to the fact that there were no or a very low act

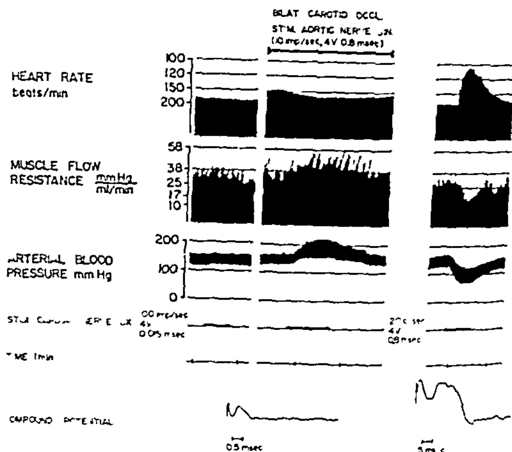


Fig. 2. Effects of stimulation of medullated (left and middle panel) and non-medullated fibres (right panel) in the main right cardiac nerve on blood pressure, heart rate and flow resistance in calf muscle. The evoked potentials recorded proximally from the same nerve are also shown. Note the augmented responses to stimulation of the medullated fibres when the buffering influence from arterial baroreceptors are largely removed (carotid occlusion) and a constant inhibitory restraint is placed on the vasomotor centre by stimulation of the aortic nerve (middle panel).

2. *Circulatory responses to stimulation of the myelinated fibre groups*

From the data presented in Fig. 1 it was possible to choose such stimulation characteristics that the "low threshold" medullated fibre group was selectively and maximally stimulated. Usually 4 V, 0.01—0.02 ms and 60—80 imp/s were chosen as stimulation characteristics.

If the stimulation was applied when the carotid arteries were unclamped and the carotid sinus baroreceptors thus allowed to display their full buffering influence no or only very small circulatory responses were induced. They consisted of slight increases of blood pressure, heart rate and calf muscle and renal flow resistances. Records from one experiment are illustrated in Fig. 2, left panel and the result of all stimulations in this series are summarized in Table I A.

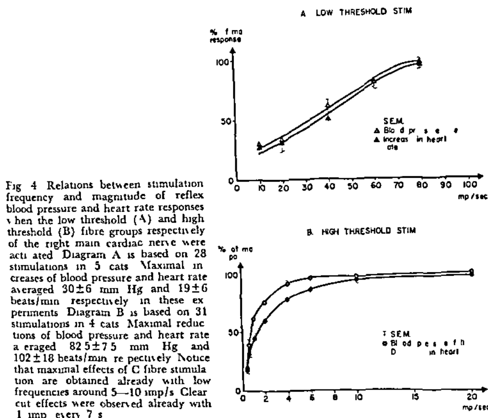


Fig 4 Relations between stimulation frequency and magnitude of reflex blood pressure and heart rate responses when the low threshold (A) and high threshold (B) fibre groups respectively of the right main cardiac nerve were activated Diagram A is based on 28 stimulations in 5 cats Maximal increases of blood pressure and heart rate averaged 30±6 mm Hg and 19±6 beats/min respectively in these experiments Diagram B is based on 31 stimulations in 4 cats Maximal reductions of blood pressure and heart rate averaged 82 5±7 5 mm Hg and 102±18 beats/min respectively Notice that maximal effects of C fibre stimulation are obtained already with low frequencies around 5—10 imp/s Clear cut effects were observed already with 1 imp every 7 s

tance) was elicited by low intensity cardiac nerve stimulation No explanation for this diverging result can at present be given

In 5 expts the relation between rate of stimulation of the medullated fibres in the cardiac nerve and the reflex blood pressure and heart rate responses were analyzed The results of these experiments are demonstrated in Fig 4 A It should be pointed out that because of the rather small maximal responses obtained with this type of stimulation the calculations of the magnitude of reflex responses in terms of per cent of maximum will inevitably suffer from certain inaccuracies and the exact course of the curve is consequently difficult to establish It was evident from the present experiments however that clearcut responses were obtained first when the stimulation frequency was around or above 10 imp/s while maximal effects were obtained with stimulation rates around 80 imp/s The blood pressure and heart rate response curves seem to have an essentially similar course

In two experiments the impulse traffic in the right cervical vagus nerve was blocked by cooling the nerve to 0° C This procedure totally eliminated all responses to cardiac nerve stimulation irrespective of intensity duration and frequency of stimulation The circulatory responses to cardiac nerve stimulation are therefore

clearly of reflex origin and the afferent pathway of this reflex run in the vagus nerve and not in e.g. cardiac sympathetic fibres (cf. Peterson and Brown 1971).

In 3 expts. where a group II of medullated fibres could be identified on the oscilloscope no circulatory responses were observed which could be correlated to the activation of this fibre group. From the values for conduction velocities it seems probable that these fibres constitute vagal efferent fibres to the heart (cf. Daly and Evans 1953, Jewett 1964).

3. Circulatory responses to stimulation of non-myelinated fibres

In all 34 expts. high intensity stimulation (4 V, 1—2 ms) produced a bradycardia, a blood pressure fall and a vasodilatation both in the calf muscles and in the kidney regardless of whether the buffering influences from the carotid sinus baroreceptors were eliminated or not (Fig. 2 and 3, right panels). The circulatory effects to stimulation of this fibre group have recently been studied in detail by Öberg and White (1970) and were therefore not subjected to a further analyzes in the present study. The inhibitory influence from these non-medullated afferents thus seem to have a particularly strong impact on the heart rate and the renal vascular bed. It deserves to be pointed out once more that indeed very powerful inhibitory responses can be produced by stimulation of cardiac nerve. For example a maximal activation of the nonmedullated fibre group was found to reduce the heart rate by 50—75 % within a few beats and sometimes even to produce at total heart standstill for a few s.

In 4 cats the relation between rate of stimulation of the non-medullated fibres and reflex heart rate and blood pressure responses were analyzed (Fig. 4 B). A stimulation frequency as low as one impulse every 7 s was found to elicit a clearcut depressor response. 70—80 % of the maximal responses were induced by stimulation rates around 2 imp/s and maximal responses were obtained with rates around 5—10 imp/s. The blood pressure response curve seems to have a somewhat steeper course than that for heart rate responses. The circulatory responses to C fibre stimulation was found to diminish again if the stimulation frequency was increased above 20—40 imp/s. This may at least partly be due to an increasing influence from the simultaneously activated excitatory medullated fibres when the stimulation rate was high.

Discussion

The present study has shown that the main right cardiac nerve in the cat contains 3 fibre groups which can be separated because of differences in conduction velocities and stimulation thresholds. Two of the groups seem to consist of medullated fibres while the third group is composed of non-medullated C fibres.

Histological analyses of the fibre composition in the cardiac nerve (e.g. Jansch and Zotterman 1948) have similarly revealed the presence of both medullated and non-medullated fibres. From such studies there seems to be a very small number of medullated fibres of the A delta group with fibre diameters between 2.8 and 7 µm and which probably corresponds to the group I in the present study. There is evidently also a fairly limited number of thinner medullated fibres with diameters

between 1 4 and 2 8 μm in the cardiac nerve These fibres are probably equivalent to the presently described group II and constitute probably efferent vagal fibres to the heart (Daly and Evans 1953) The majority of the fibres in the cardiac nerve are presumably non medullated (Jarisch and Zotterman 1948 Agostino *et al* 1957) corresponding to the fibre group III in the present study

The medullated afferents in the right main cardiac nerve must reasonably emanate from a variety of different receptors in the cardio-pulmonary area such as atrial receptors and ventricular pressure receptors (see below) A low intensity electrical stimulation of the cardiac nerve therefore implies a simulataneous and indiscriminate activation of several different afferent systems That notwithstanding it seems quite clear from the present results that an increased impulse activity in these medullated afferents influences the circulatory system to only a minor extent at least in the cat and then in an excitatory direction Clearcut although rather moderate *increases of blood pressure heart rate and flow resistances in response to low intensity cardiac nerve stimulation* could thus be obtained only when the buffering influences from other receptor areas were minimized and the level of activity in the medullary cardiovascular centres was at the same time maintained low From these observations it seems safe to conclude that the very marked depressor reflexes of the Bezold—Jarisch type which can be elicited by stimulations of endings within the heart are not initiated from receptors firing in medullated afferents such as atrial receptors or ventricular pressure receptors Such reflex depressor responses *must be ascribed excitation of endings with non medullated afferents* since marked inhibitory responses to cardiac nerve stimulation were obtained only in case the stimulation intensity was high enough to activate the C fibres

The importance of receptors signalling in non medullated afferents for the emergence of the Bezold—Jarisch reflex has been pointed out earlier by several workers (Jarisch and Zotterman 1948 Zotterman 1948 Sleight and Widdicombe 1965 and Öberg and Thoren 1972) According to these authors the attached receptors are to a large extent localized in the walls of the heart ventricles probably *both in the epicardium and at varying depths in the myocardium* An obviously large population of these receptors respond to a distension of the ventricles and also to administration of various drugs like nicotine and veratrum alkaloides (Öberg and Thoren 1972) Since such procedures are known to induce powerful reflex depressor responses of the same type as obtained with high intensity cardiac nerve stimulation it seems reasonable to assume that at least a large portion of the non medullated afferents in the main cardiac nerve arises from these endings—These receptors normally display a very low spontaneous activity (Öberg and Thoren 1972) but even a fairly moderate increase of receptor discharge from *e g 0 to 1 imp/s is* capable to induce pronounced reflex responses corresponding to approximately 50 % of maximum (*cf* Fig 4) provided of course that there is a simultaneous activation *of all or the majority of the endings*

It would be of interest to define the receptors with medullated afferents which upon activation are capable to elicit clearcut circulatory responses of an excitatory

nature As mentioned several types of endings must be considered as possible candidates If the impulse traffic in the cardiac nerve is recorded the dominating activity usually arises from atrial receptor afferents (e g Jarisch and Zotterman 1948 Thorén unpublished observations) Occasionally one or a few lung inflation receptor afferents can also be identified (Thorén unpublished data) However activation of these latter afferents can in all probability not be responsible for the presently observed circulatory responses to low intensity stimulation of the cardiac nerve since excitation of lung inflation receptors is said to produce besides a tachycardia a vasodilatation in the muscle (Daly and Robinson 1968) Such a response pattern was never observed in the present study It seems likely that afferents from so called ventricular pressure receptors (Paintal 1955) also travel in the cardiac nerve The number of such receptors seems however to be very small in the cat (Paintal 1955) and their afferents must therefore constitute a very small part of the medullated fibre group in the cardiac nerve Consequently these afferents probably contribute very little to the circulatory responses to cardiac nerve stimulation The pressor responses to cardiac nerve stimulation may suggest activation of afferents from chemoreceptors located in the heart or adjacent structures. However to the authors knowledge such afferents have never been described in the cardiac nerves. Therefore since the number of atrial receptors seem to be relatively large while there is a limited number of medullated fibres in the cardiac nerve (Jarisch and Zotterman 1948) it seems reasonable to assume that the great majority of these afferents emanate from atrial receptors The circulatory responses to low intensity stimulation of the cardiac nerve should therefore mimic those obtained when atrial receptors are activated

That atrial receptors located in the pulmonary vein atrial junctions exert an excitatory influence particularly with regard to heart rate has been shown in experiments on dogs by Ledsome and Linden (1964) However this excitation does not seem to imply a positive inotropic influence on the heart (Furnival Linden and 1971) The skeletal muscle vascular bed seems to be essentially uninfluenced when atrial receptors are stimulated (Carswell Hainsworth and Ledsome 1970) while under such circumstances a reduction of the tonic sympathetic discharge to the kidney has been reported (Karim et al 1971) Corresponding receptors in the right atrium seems to have essentially similar effects (Kappagoda Linden and Snow 1972) Somewhat different results are reported by Edis Donald and Shepherd (1970) who found a clearcut reflex vasodilatation in the hind limbs on distension of the pulmonary vein—atrial junctions Furthermore they report that a reflex tachycardia was observed only if the heart rate was initially low

The results of the present stimulations of the non medullated fibres in the cardiac nerve differ from the abovementioned findings in that respect that a more generalized excitation of the medullary cardiovascular centres was obtained This resulted in, besides a tachycardia also a clear blood pressure rise and a vasoconstriction in both the skeletal muscle and the kidney These discrepancies may be due to species differences or to the fact that in the present study certain measures (e g carotid

occlusion and afferent stimulation of the aortic nerve) were deliberately taken to promote the appearance of the excitatory reflex responses It is of course also possible that the presently observed increased vasoconstrictor discharge to the vascular beds was produced by an activation of afferents from other types of endings than atrial receptors

The fairly small circulatory effects caused by even a maximal activation of the medullated afferents in the cardiac nerve suggest that the attached receptors such as atrial receptors play a minor role in the regulation of the circulation The tonic discharge from these endings may therefore not be primarily directed to the bulbar cardiovascular centres but possibly towards other central s ructures involved in *e g* the homeostatic control of blood volume Hormonal mechanisms rather than vasomotor fibres seem to constitute the main efferent pathway in this control system (*cf* Gauer Henry and Behn 1970)

In contrast extremely powerful circulatory responses can be elicited from cardiac receptors firing in non medullated afferents These endings normally show a low spontaneous activity with no cardiac rhythm (Öberg and Thoren 1972) Therefore when activity in cardiac afferents is recorded with conventional electrophysiological techniques the impulse traffic in these afferents appears to be insignificant and is indeed often completely obscured by the high frequency rhythmic discharged in the medullated fibres from *e g* atrial receptors It is therefore not surprising that the importance of the non medullated cardiac afferents for producing powerful circulatory responses as clearly demonstrated by Jarisch and Zotterman (1948) and Zotterman (1948) has been somewhat underrated in current literature and that most reflex circulatory responses of cardiac receptor origin such as *e g* the Bezold—Jarisch reflex have erroneously been ascribed to an increased impulse activity in medullated afferents

This work was supported by the Swedi h Medical Research Council Project No B/0-14X 644 06A and by the Medical Faculty University of Goteborg

Thanks are due to Mrs Gertrud Karlsson and Mrs Eva Cari Einarsson for able technical assistance

References

AGOSTINO E J E CHINNOCK M DE BURGH DALY and J G MURRAY Functional and histological studies of the vagus nerve and its branches to the heart lungs and abdominal viscera in the cat *J Phy ol* (Lond) 1957 *135* 182—205

ANUFRIEW W N Die Herznerven der Katze *Z Anat Entw ckl Gesch* 1928 *86* 639—654

AVIADO D M and C F SCHMIDT Reflexes from stretch receptors in blood essels heart and lungs *Physiol R v* 1955 *35* 247—300

CARSWELL F R HAINSWORTH and J R LEDSOME The effects of distension of the pulmonary vein atrial junction upon peripheral vascular resistance *J Physiol* (Lond) 1970 *207* 1—14

DALY M DE B and D H L EVANS Functional and histological changes in the agu nerve of the cat after degenerati e section at various levels *J Phy iol* (Lond) 1953 *120* 579—595

DALY M DE B and B H ROBINSON An analysis of the reflex systemic vasodilator response elicited by lung inflation in the dog *J Phys l* (Lond) 1968 *195* 387—406

DAWES G S and J H COMROE Chemo eflexes from the heart and lungs *Physiol Rev* 1954 *34* 167—201

EDIS A J D E DONALD and J T SHEPHERD Cardiovascular reflexes from stretch of pulmonary vein atrial junctions *Circulat Res* 1970 *27* 1091—1100

FURNIVAL C M R J LINDEN and H M SNOW Reflex effects on the heart of stimulating left atrial receptors *J Physiol* (Lond) 1971 *218* 447—463

GAUER O H J P HENRY and C BEHN The regulation of extracellular fluid volume *Ann Rev Physiol* 1970 *32* 547—595

JARISCH A and Y ZOTTERMAN Depressor reflexes from the heart *Acta physiol scand* 1948 *16* 31—51

JEWETT D L Activity of single efferent fibres in the cervical vagus nerve of the dog with special reference to possible cardioinhibitory fibres *J Physiol* (Lond) 1964 *175* 321—357

JONES J V The afferent pathway of the Bezold reflex the right vagal branches in cats *Brit J Pharmacol* 1953 *8* 352—355

KAPPAGODA C T R J LINDEN and H M SNOW A reflex increase in heart rate from distension of the junction between the superior vena cava and the right atrium *J Physiol* (Lond) 1972 *220* 177—197

KARIM F C KIDD C M MALPUS and P E PENNA The effects of stimulation of the left atrial receptors on sympathetic efferent nerve fibres *J Physiol* (Lond) 1971 *213* 38—39 P

LEDSOME J R and R J LINDEN A reflex increase in the heart rate from distension of the pulmonary vein atrial junctions *J Physiol* (Lond) 1964 *170* 456—473

NEIL E and Y ZOTTERMAN Cardiac vagal afferent fibres in the cat and frog *Acta physiol scand* 1950 *20* 161—165

NONIDEZ J F Identification of the receptor areas in the venae cavae and pulmonary veins which initiate reflex cardiac acceleration (Bainbridge's reflex) *Amer J Anat* 1937 *61* 203—231

OBERG B and P THOREN Circulatory responses to electrical stimulation of medullated and non medullated afferents in the cardiac nerve *Proc XXV Intern Cong Physiol* Munich 1971 *1958* 424

OBERG B and P THOREN Studies on left ventricular receptors signalling in non medullated vagal afferents *Acta physiol scand* 1972 *85* 164—173

OBERG B and S WHITE Circulatory effects of interruption and stimulation of cardiac vagal afferents *Acta physiol scand* 1970 *80* 383—394

PAINTAL A S A study of ventricular pressure receptors and their role in the Bezold reflex *Quart J exp Physiol* 1955 *40* 348—363

PAINTAL A S Vagal afferent fibres *Ergebn Physiol* 1963 *52* 74 -156

PETERSON D F and A M BROWN Pressor reflexes produced by stimulation of afferent fibres in the cardiac sympathetic nerves of the cat *Circulat Res* 1971 *28* 605—610

SALISBURY P F C E CROSS and P A RIEBEN Reflex effects of left ventricular distension *Circulat Res* 1960 *8* 530—534

SLEIGHT P and J C WIDDICOMBE Action potentials in fibres from receptors in the epicardium and myocardium of the dog's left ventricle *J Physiol* (Lond) 1965 *181* 235—258

ZOTTERMAN Y Afferent impulses from the heart *Nord Med* 1948 *38* 1182—1189

Acta physiol scand 1973 87 133—137

From the Department of Medical Chemistry University of Turku Finland

Metabolism of Fracture Callus of Rat in vitro
I Oxygen Consumption and Lactic Acid Production

By

R PENTTINEN

Received 5 May 1972

Abstract

PENTTINEN R *Metabolism of fracture callus in vitro I Oxygen consumption and lactic acid production* Acta physiol scand 1973 *87* 133—137

Compared with the rat bone the oxygen uptake by the slices of callus is low during the proliferation of the cartilage in the callus and even lower during the active ossification The production of lactate by the slices is equal to that of rat bone These two parameters of the energy metabolism expressed per whole callus are maximal on the 9th day after the fracture corresponding to the degeneration and calcification of the cartilage Calculated on the weight basis both parameters decline during the healing The transition from cartilaginous to ossifying callus is characterized by a drop in the ratio of lactate produced to oxygen consumed It is concluded that the energy metabolism in regenerating bone is mainly anaerobic thus resembling the granulation tissue

Enzymes of the tricarboxylic acid and pentose phosphate cycles have been demonstrated in callus tissue (Balogh and Hajek 1965 Takada 1966) Histochemical studies suggest however that the energy metabolism of fracture callus may be shunted to more anaerobic pathways than that of intact bone (Gudmundson and Semb 1971) This concept is supported by the subunit pattern of cartilage LDH isoenzymes (Kunin and Krane 1965) No significant change in the arteriovenous pO_2 difference of nutrient vessels of a fractured bone was found during the healing (Laurnen and Kelly 1969) indicating a low O_2 uptake by the callus

To our knowledge no related data on the energy metabolism of isolated callus tissue have been reported The present experiments were carried out to study the viability of callus slices and to follow the metabolism of the slice at the various phases of healing

Material and Methods

Preparation of samples Tibias of 6—7 week male rats of Wistar strain (132±9 g ±S E n = 43) were fractured bilaterally (Penttinen *et al* 1972 a) and the rats killed at 3 5 7 9 11 14 and 21 days after the fracture The calluses were prepared immediately in the cold room weighed placed in fresh ice cold medium and mounted in a polytheylene tube (length 20 mm I D 6 mm O D 10 mm) cut with a microtome (E Leitz Wetzlar West Germany type 20 827) into 200 μm thick slices which were rinsed with the medium to remove blood and marrow cells About 60—90 min elapsed during the preparation of the samples

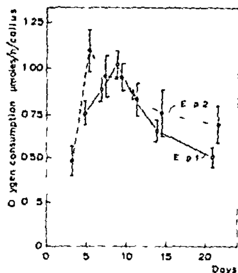


Fig 1

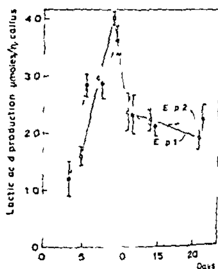


Fig 2

Fig 1 Oxygen consumption of a single sliced callus from two similar series of experiments The means with their standard errors (vertical bars) are given (n = 6—8)

Fig 2 Lactic acid production of sliced callus (cf legend of Fig 1)

Incubation of callus slices The slices of each callus (weighing 919±9 mg ±S E) were incubated in one Warburg manometer flask (Hormuth Inh W E Vetter Heidelberg West Germany) at +37 C in 3 0 ml Krebs Ringer phosphate buffer with 27 4 mM glucose/l (Umbreit et al 1957 Lampiaho and Kulonen 1967) and 0 1 mg/ml ampicillin (Doctacillin Astra, Sweden) Six to eight calluses were incubated simultaneously Carbon dioxide was absorbed with 0 1 ml of 5 % (w/v) KOH in the centre well In preliminary experiments the slices consumed oxygen at a constant rate for at least 9 h After 6 h incubation the flasks were cooled in crushed ice and 0 2 ml trichloroacetic acid (TCA) solution (100 g to 100 ml distilled water) was added The mixture was transferred into centrifuge tube the incubation flasks washed once with 1 0 ml of 5 (w/v) TCA solution each combined sample homogenized for 2×5 s with an Ultra Turrax homogenizer (Type TI 18/2 N Jahnke & Kunkel Staufen West Germany) and centrifuged at 10 000 g for 20—30 min The sediments were washed once with 1 0 ml of 5 TCA Lactic acid was determined from the supernatants according to Barker and Summerson (1941) The variation coefficient of the lactate determination method was ±4 6 %

Results

Oxygen consumption Two series of experiments were carried out in similar conditions (Fig 1) The slices of a single whole callus used oxygen maximally between the 7th—11th days after the fracture Calculated either per wet or dry weight the O_2 consumption declined during the observation period Between the 11th and 21st days i e during the ossification of callus the oxygen uptake of the sliced callus decreased by 50 % or more of the peak value

Production of lactate The slices of a single whole callus produced 1 3—4 0 μmol lactate per hour (Fig 2) Production was at the highest on the 9th day after the fracture Calculated per wet or dry weight it decreased evenly during the observation period The ratio of the lactic acid production to the oxygen uptake plotted against the time after the fracture forms a biphasic curve because of a transient minimum on the 11th day (Fig 3)

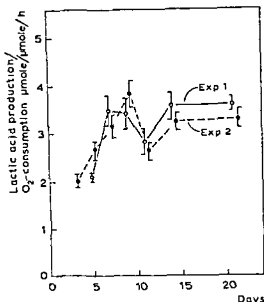


Fig 3 Ratio of the lactic acid production to the oxygen consumption The original data are given in Fig 1 and 2

Discussion

Oxygen consumption of callus slices The studies by Laurnen and Kelly (1969) on arteriovenous pO_2 pCO_2 and pH differences indicate a low O_2 consumption by dog tibial callus which is in agreement with this work The observed result 0 3—1 8 µl O_2/h/mg dry weight of the callus is equal or less than values reported for rat femur (Flanagan and Nichols 1965) The calculation per dry weight (Dittmer and Grebe 1958) is not adequate for tissues which contain variable amounts of minerals Assessed per DNA indicating the number of cells (Penttinen *et al* 1972b) the consumption varied between 0 6—2 2 µmol O_2/h/mg DNA However the content of DNA per sample weight varies also considerably (Nichols *et al* 1969) The present results are referred with data available on hard connective tissues in Table I

Compared with values reported for slices of brain epithelial tissues muscle or liver (Dittmer and Grebe 1958) callus consumes oxygen at a very low rate During the cartilaginous phase the respiration is equal to that of human and rat bone or rabbit costal cartilage but below that of rat epiphyseal plate or mouse bone and decreases during the ossification Calculated against DNA more oxygen is consumed by slices of callus than by slices of human bone Different methods for extraction of DNA (Flanagan and Nichols 1965 Penttinen *et al* 1972b) may give different yields of DNA from bone or callus Tissue wet weight was therefore used in the expression of the results in Table I

Production of lactic acid Considerable amounts of lactate were produced although the experiments were carried out in aerobic environment Slices of cartilaginous callus and intact rat bone produced equal amounts of lactate per wet weight Decreased activities of glucose 6 phosphate dehydrogenase and isocitric dehydrogen

TABLE I Oxygen consumption and lactate production in some hard connective tissues The values have been recalculated to μmoles h/100 mg wet weight Where necessary the authors data on dry weight vs wet weight have been applied

Tissue	O_2-Consumption	Lactate production
rabbit costal cartilage	0 46	–
rat epiphyseal plate	–	2 [illegible]
mouse femur and tibia	appr 1 1	appr 2 6
rat femur[a]	–	0 [illegible]0
mouse femur and tibia[b]	–	3 09 – 3 36
human iliac crest	0 91	1 53
rat femur and tibia	1 07	0 [illegible]8 – 1.58
mouse femur	2 35	1 88
rat femur and tibia	–	0 58
rat callus present results	0 16 – 0 94	0 6f – 1 52

Laskin and Sarnat 1953 Leg Larsen 1950 [a] Borle et al 1960 [b] Deiss et al 1962 [c] Vaes and Nichols 1962 Flanagan and Nichols 1965 Murphy 1969 Nichols et al 1969

ase have been demonstrated in the chondrocytes during the hypertrophy of the cartilage cells in callus (Balogh and Hajek 1965) which is in agreement with the decreased respiration of the callus cells after the ninth day of healing

Calculated per DNA both consumption of oxygen and production of lactate show maximal values on the 9th day after the fracture and decline thereafter The lactate production correlates significantly only to the consumption of oxygen and to the wet weight r = 0 784 and 0 580 respectively $p < 0.001$) The correlations of the O_2-consumption or the lactate production to the dry weight amount of proteins carbohydrates or minerals are not significant (to be published)

The low oxygen consumption and the considerable aerobic production of lactate support the histological findings of Takada (1966) and Gudmundson and Semb 1971 These results as well as the manometric measurements of Lampiaho and Kulonen (1967 or Niinikoski (1969) on granulation tissue suggest that the energy metabolism of various regenerating connective tissue cells is anaerobic

I am grateful to Professor E Kulonen M D for his advice This study has been supported by a personal grant from the Emil Aaltonen Foundation and institutional grants from the Sigrid Juselius Foundation

References

BALOGH K JR and J V HAJEK Oxidative enzymes of intermediary metabolism in healing bone fractures A histochemical study Amer J Anat 1965 116 479—448
BARKER S B and W H SUMMERSON The colorimetric determination of lactic acid in biological material J biol Chem 1941 138 535—554
BORLE A B N NICHOLS and G NICHOLS JR Metabolic studies of bone in vitro I Normal bone J biol Chem 1960 35 1206—1210
DEISS W P JR L B HOLMES and C C JOHNSTON JR Bone matrix biosynthesis in vitro I Labeling of hexosamine and collagen of normal bone J biol Chem 1962 237 3555—3559
DITTMER D S and R M GREBE Handbook of respiration W B Saunders Company Philadelphia and London 1958

EEG-LARSEN N An experimental study on growth and glycolysis in the epiphyseal cartilage of rats *Acta physiol scand* 1956 *Suppl 128*

FLANAGAN B and G NICHOLS JR. Metabolic studies of human bone *in vitro* I Normal bone *J clin Invest* 1965 *44* 1788—1794

GUDMUNDSON C and H SEMB Enzyme studies of fractures with normal and delayed union *Acta orthop scand* 1971 *42* 18—27

KUNIN A S and S M KRANE The effect of dietary phosphorus on the intermediary metabolism of epiphyseal cartilage from rachitic rats. *Biochim biophys Acta* (Amst) 1965 *107* 203—214

LAMPIAHO K and E. KULONEN Metabolic phases during the development of granulation tissue *Biochem J* 1967 *105* 333—341

LASKIN D M and B G SARNAT The metabolism of fresh transplanted and preserved cartilage *Surg Gynec Obstet* 1953 *96* 493—499

LAURNEN E L and P J KELLY Blood flow oxygen consumption carbon-dioxide production and blood calcium and pH changes in tibial fractures in dogs *J Bone Jt Surg* 1969 *51* A 298—308

MURPHY H Citrate metabolism in the osteopetrotic bone of the grey lethal mouse *Calc Tiss Res* 1969 *3* 176—183

NICHOLS G JR B FLANAGAN and J VAN DER SLUYS VEER, Distortions of bone cell metabolism in uremia and their cause *Arch intern Med* 1969 *124* 530—538

NIINIKOSKI J Effect of oxygen supply on wound healing and formation of experimental granulation tissue *Acta physiol scand* 1969 *Suppl 334*

PENTTINEN R J NIINIKOSKI and E KULONEN Hyperbaric oxygenation and fracture healing A biochemical study with rats *Acta chir scand* 1972 a. *138* 39—44

PENTTINEN R J NIINIKOSKI J RANTANEN and E KULONEN The effects of hyperbaric oxygenation and reduced air pressure on the nucleic acid contents of healing fractures A biochemical study in rats *Acta chir scand* 1972 b *138* 269—274

TAKADA K Enzyme histochemistry in bone tissue II Histochemical detection of hydrolytic and oxidative enzymes in callous tissue *Acta histochem* (Jena) 1966 *23* 53—70

UMBREIT W W R. H BURRIS and J F STAUFFER *Manometric Techniques* 1957 1—338 Minneapolis Burgess Publ Co

VAES G and G NICHOLS JR. Effects of a massive dose of parathyroid extract on bone metabolic pathways *Endocrinology* 1962 *70* 546—555

Acta physiol. scand. 1973. 87. 138—144
From the Institute of Medical Physiology, Dept. A, University of Copenhagen, Denmark

Transcapillary Transport of D- and L-Glucose in Isolated Skeletal Muscle

By
C. CRONE

Received 20 May 1972

Abstract

CRONE, C. *Transcapillary transport of D- and L-glucose in isolated skeletal muscle.* Acta physiol. scand. 1973. 87. 138—144.

The study was performed to clarify whether D-glucose traverses muscle capillary walls faster than L-glucose. The transcapillary transport of D-glucose was slightly faster than that of L-glucose in experiments at 37° C. The difference in transport rate persisted when the experiments were carried out at 4° C. This indicates that the faster transport of D-glucose is not due to facilitated transport involving enzymatic steps. It is proposed that the ground matrix of the interendothelial cleft (and of the extravascular space) gives the D-form of glucose a slight diffusional advantage over the L-form. A pore effect cannot be completely ruled out.

There have been many indications that D-glucose passes from blood into brain by a process of facilitated diffusion (Crone 1965, LeFevre and Peters 1966, Buschiazzo *et al.* 1970, Oldendorf 1971, Bidder 1968, Gilboe and Betz 1970). However, the anatomical location of the glucose transport system is so far unknown. It has been speculated that the plasma membranes of the endothelial cells in the brain are involved and it might therefore be of interest to know whether other capillaries, for example muscle capillaries, show at least traces of the same mechanism.

Such a study could be performed by comparing transport rates of the isomers D- and L-glucose in muscle capillaries as it has been done in brain (Crone and Thompson 1972). In contrast to brain, muscle capillaries have a significant leak or passive diffusion path between endothelial cells. A diffusion channel between endothelial cells does not discriminate between isomers and tends to reduce any differences in transport rate between the 2 forms. In brain, where the leak between endothelial cells is insignificant, D-glucose is transported 8–10 times faster than L-glucose. In muscle one might expect only a slight preference for D-glucose because the possible selective facilitated D-glucose transport is put on top of a substantial common transport via the interendothelial pores.

Although the possibilities for obtaining a clear answer were not the best experiments were nevertheless carried out The main finding was that there was in fact a slightly faster transcapillary transport of D glucose in experiments carried out at 37° C but this preference also exists when the experiments were carried out at 4° C where one would have expected facilitated diffusion processes to be virtually arrested

Methods

Cats of medium size were used They were anesthetized with chloralose-ether After isolation of the gastrocnemius muscle but with the vascular connection still present the cat was heparinized The perfusion system which permitted a Ringer dextran perfusion of isolated gastrocnemius muscle has been described in detail by Crone and Garlick (1970) Its main points are a temperature stabilized reservoir containing Ringer-dextran fluid in equilibrium with 95 % O_2—5 % CO_2 Albumin was added to give a concentration of 20 g/liter The perfusion fluid was circulated through water jacketed tubes by means of a roller pump A polyethylene catheter was introduced into the artery to the gastrocnemius muscle The vein draining the muscle was also catheterized so as to permit rapid collection of effluent fluid The fluid was not recirculated

Perfusion flows varied between 10 7 and 32 8 ml/100 g/min Experiments were carried out at 2 temperatures 37 C and at 4 C The perfusion fluid contained 100 mg % D glucose in the period before the injection some time before the experiment it was substituted with Ringer s fluid containing 10 mg % glucose After the experiment perfusion was continued with 100 mg % D glucose in the perfusate until ready for the next experiment. 2—4 expts were carried out on each muscle When experiments were carried out at 2 temperatures either this was done on separate days (and therefore on separate muscles) or on the same day allowing conditions to stabilize at the new temperature It was regularly observed that the perfusion pressure rose when the temperature was lowered

The perfusion pressures were measured with a Statham strain gauge transducer connected with suitable amplifier and recorded on a Brush two-channel ink writer

The experiments were carried out with Indicator Diffusion technique (Crone 1963 a) consisting in a square wave injection of a mixture of a non permeable reference solute (Evans Blue Dye) and L-glucose 1 ^{14}C and D glucose 6 T supplied from the Radiochemical Centre Amersham

The volume of the injection solution was about 0 2—0 25 ml It always contained serum about 0 05 ml in order to ensure protein binding of Evans Blue Dye 0 05 ml of a 5 % solution (Warner Chilcott) The specific activities of the radioactive tracers were 2 7 mCi/mg for tritiated D glucose and 16 2 μCi/mg for ^{14}C L glucose 25—50 μCi of T D glucose and 5—7 μCi of ^{14}C L glucose were injected The activity of the injection solution was determined after an aliquot of it was added to a volume of the Ringer s fluid Thus as well the activity of the injectate and of the collected samples were determined on the same material which is important in order to reduce differences in quenching After taking an aliquot for determination of Evans Blue Dye the samples were precipitated with 5 % trichloro-acetic acid to get rid of the Evans Blue Dye and the radioactivity was determined by adding 0 3 ml of the supernatant to 15 ml of Bray s scintillation mixture (Bray 1960) Counting took place in a Packard Liquid Scintillation Spectrometer (model 3003) with appropriate gains and window settings The Evans Blue Dye concentration was determined on a Beckmann Spectrophotometer model DU at 650 nm wavelength

For sampling of the effluent perfusion fluid a slowly moving kymograph was used carrying small glass vials The sampling time for each sample was 2—5 s depending on the flow rate

Calculations The extraction (E) the fraction of the injected tracer which left the capillaries during a single transit was determined as $E = (a-b)/a$ where a is the concentration of Evans Blue Dye expressed relative to the concentration in the injection mixture and b is the concentration of the labelled D or L glucose expressed relative to the concentration in the injection mixture

As the perfusion flow (F) was known the product of permeability and capillary surface area (PS product) could be calculated according to Renkin (1959) as $PS = -F \times \ln(1-E)$ Permeability ratios were calculated as $\ln(1-E_1)/\ln(1-E_2)$ taking advantage of the cancelling out of the unknown surface area when ratios between 2 solutes are determined (Crone 1963 b) under the same circumstances (both being injected together)

TABLE I Extractions and PS products of L- and D glucose at 37 C in muscle capillaries

Exp no	Perfusion pressure mm Hg	Rate of perfusion ml/100 g/min	Extraction		PS-product ml/100 g/min	
			L	D	L	D
9a	110	17 3	11 6	12 8	2 13	2 37
9c	130	17 3	7 1	9 8	1 28	1 8
15a	85	25 4	25 0	26 4	7 31	7 9
15b	110	32 8	18 7	20 3	6 79	7 44
16a	80	10 7	19 6	20 9	2 34	2 51
16b	85	15 4	16 8	18 1	2 83	3 08
19c	78	19 5	25 2	27 1	5 66	7 74
20b	68	20 8	7 0	9 6	1 53	2 14
21c	30	14 8	30 2	32 2	5 37	5 5
21d	30	14 2	26 8	29 2	4 43	4 90

TABLE II Extractions of L- and D glucose at 4 C in muscle capillaries

Exp no	Perfusion pressure mm Hg	Rate of perfusion ml/100 g/min	Extraction		PS product ml/100 g/min	
			L	D	L	D
17b	128	20 9	5 3	7 3	1 14	1 8
18a	153	17 3	4 7	7 2	0 83	1 29
18b	145	17 3	3 5	6 1	0 62	1 09
18c	125	11 9	6 5	10 0	0 80	1 25
19b	110	19 5	3 6	5 3	0 63	1 06
20a	90	19 0	7 6	8 1	1 50	1 61
21a	30	14 8	13 1	16 2	2 08	2 6
21b	90	14 8	17 1	17 5	2 8	85

Results

20 injection expts were carried out on 8 different gastrocnemius muscles The experiments fell in 2 groups according to whether the experiment was performed at 37° C or at 4° C 10 expts were made at each level of temperature

The reason for performing experiments at 2 temperatures was that if a facilitated transendothelial transport of D glucose occurred it would disappear or be strongly reduced at 4° C and the expectation was that one would observe similar transport rates of D and L glucose at low temperature

The results are shown in Table I and II It is seen that in fact the extraction of D glucose was always higher than that of L glucose but that the differences were rather small

The PS products are clearly higher in the experiments at high temperature than at 4° C There is a tendency for the PS products to increase with increase in flow and the values stabilize at values about 5—7 ml/ 100 g/min in the 37° C expts The endency to increase is much less pronounced in the 4° C expts where the values

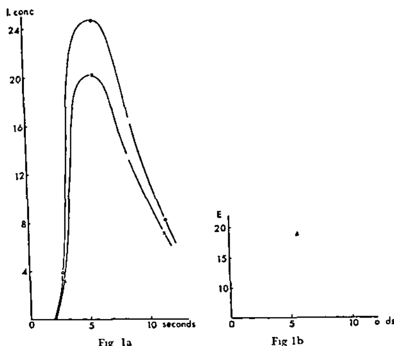


Fig 1a Fig 1b

Fig 1 Typical venous outflow time concentration curves for E ans Blue and labelled D glucose from an isolated perfused gastrocnemius muscle (Exp no 16 b) Right part of graph shows corresponding values for fractional extraction of D glucose and L-glucose in each sample The time concentration curve for L-glucose is not drawn on the left curve because it lies so close to the D glucose curve Symbols on right graph *triangles* D glucose *crosses* L-glucose The experiment was conducted at 37 C

are rather stable throughout about 1 5—2 5 ml/100 g/min The range of flows was somewhat more limited in the 4° C expts but this seems not to explain the greater constancy of the PS products A true diffusion (or barrier) limited situation is reached at lower flows when the temperature is low Fig 1 a & b shows the time concentration curves and the calculated extractions of D and L-glucose as they were obtained in a typical experiment conducted at 37° C When comparing the values of flows and PS products with other values in the literature it should be remembered that in the present experiments the perfusion fluid does not contain red cells and that the flow values therefore are comparable to blood flows twice as high The dimensionless figure PS/F which is an index of whether a barrier limited situation is present (Goresky 1970 Levitt 1970) was below 0 3 in almost all experiments At this value of PS/F one should be in the barrier limited condition

The extractions of L and D glucose were higher at 37° C than at 4° C at similar flow rates

The average ratio between permeabilities of D and L glucose (P_D/P_L) was 1 2 at 37° C and 1 4 at 4° C This difference is not significant ($p > 0\ 1$) In no instance was the ratio below 1 (Table III)

TABLE III Permeability ratios of D- and L-glucose in muscle capillaries (P_D/P_L)

	37° C	4° C
P_D/P_L	1 18 (n = 10) (S D = 0 15)	1 43 (n = 9) (S D = 0 6)

Table I and II show that the perfusion pressures were higher in the low temperature experiments This is to be expected in view of the temperature dependence of viscosity A plot of the perfusion resistances (pressure per ml/100 g/min) showed that the average ratio between hemodynamic resistances at 37° C and at 4° C were about 1 4 In view of the rather great scatter of results this figure is probably not different from the ratio between viscosities of water at the 2 temperatures 2 5 according to Partington (1951)

Discussion

The experiments were performed to see whether there were any differences in the transcapillary transport of D and L-glucose It was found that the transport rate of D glucose was about 20 % higher than that of L glucose (in experiments at normal body temperature) This finding seems to corroborate the idea that the transport of D glucose across the capillary wall in muscle has a facilitated component However the results do leave some problems as to the interpretation It is clear that by far the greatest part of the D glucose transport takes place through the interendothelial gap This is seen from the fact that the glucose extractions agree with what has been found for similar hydrophilic solutes (Pappenheimer Renkin and Borrero 1951 Crone 1963 a Alvarez and Yudilevich 1969 Trap Jensen and Lassen 1971) which are not transported by facilitated mechanisms

The results of the experiments carried out at 4° C were of course unexpected according to the working hypothesis The fact that the transport rate of D glucose relative to that of L glucose is higher at 4° C speaks against the difference being due to facilitated transport of D glucose since it is known that faciliated tran port processes virtually stop at low temperatures (Sen and Widdas 1962 Balis et al 1970)

If the different transport rate of the 2 molecules is not due to transendothelial transport of D glucose then the possibility must be considered that the diffusion velocity of the 2 isomers in the interendothelial slit is different There is not much evidence to support any detailed discussion of this point Only the studies of Craig (1964) lend some support to this idea Craig found that when molecules diffuse through membranes with pores of molecular dimensions small differences in free diffusion rates are greatly amplified Thus he found that the ratio between the free diffusion coefficients of the isomers galactose and glucose was 0 01 while the ratio

between the permeabilities (expressed as the ratio between 50 % escape rates) was 1 37 when the molecules diffused through cellophane in thin film membrane diffusion

Another possible explanation is that the ground matrix which fills the pore affects the rate of diffusion of solutes with similar free diffusion coefficients It is reasonable to expect that the ground matrix in the interstitial tissue which contains hyaluronic acid as the most important structural element (Laurent 1970) extends within the interendothelial space itself although this is not known to be the case Hyaluronic acid contains D glucose residues and it cannot be completely ruled out that diffusion of D forms for this reason occurs more rapidly than of L forms However this is conjectural and further discussion must be deferred until more is known about this possibility

The permeability of the capillary membrane measured as the PS product fell with a factor of 3—4 in the experiments conducted at 4° C This is quite in accordance with the temperature dependence of diffusion Longsworth (1952) found the diffusion coefficient of D glucose at 1° C to be 3 137 $\times 10^{-6}$ cm /s while the diffusion coefficient at 37° C is 9 0 $\times 10^{-6}$ (Landis and Pappenheimer 1963) The observation that the ratio of PS products corresponds to that of the diffusion coefficients speaks against any significant change in capillary surface area with temperature

The PS products as found in the present experiments agree with values from studies of transcapillary movement in muscle Trap Jensen and Lassen (1971) found PS products for fructose at 37° C of 5 55 ml/100 g/min in the exercising human forearm in complete agreement with what has been found in the present study However Alvarez and Yudilevich (1969) found that in heart muscle the PS products were definitely higher about 20—40 ml/100 g/min for similar solutes

The changes in perfusion pressure with temperature were in accordance with the increase in viscosity of the perfusion fluid at low temperature a finding which also agrees with the postulated constancy of the capillary (and therefore arteriolar) surface area at the 2 temperatures

In brief the experiments did not support the contention that a small fraction of D glucose transport in muscle capillaries takes place via the endothelial cells The small difference in transport rate of D and L glucose is unexplained but is probably due either to the structure of the ground matrix in the interendothelial slit or to a pore effect (Craig)

References

ALVAREZ O A and D L YUDILEVICH Heart capillary permeability to lipid insoluble molecules *J Physiol* (Lond) 1969 *202* 45—58

BALIS L P LULY B A PETHICA and W WILBRANDT The temperature dependence of the facilitated transport of D(+) glucose across the human red cell membrane *J Membr Biol* 1970 *3* 83—92

BRAY J A A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter *Analyt Biochem* 1960 *1* 279—285

BUCHIAZZO I M E B TERRELL and D M REGEN, Sugar transport across the blood brain barrier *Amer J Physiol* 1970 *219* 1505—1513
CRAIG L C Differential dialysis *Science* 1964 *144* 1093—1099
CRONE C The permeability of capillaries in various organs as determined by use of the Indicator Diffusion method *Acta physiol scand* 1963 a *58* 292—305
CRONE C Does restricted diffusion occur in muscle capillaries? *Proc Soc exp Biol* (NY) 1963 b *112* 453—455
CRONE C Facilitated transfer of glucose from blood into brain tissue *J Physiol* (Lond.) 1965 *170* 103—113
CRONE C and D GARLICK The penetration of inulin sucrose mannitol and tritiated water from the interstitial space in muscle into the vascular system *J Physiol* (Lond) 1970 *210* 387—404
CRONE C and A M THOMPSON Comparative studies of capillary permeability in brain and muscle *Acta physiol scand* 1973 In press
GILBOE D D and A L BETZ Kinetics of glucose transport in the isolated dog brain. *Amer J Physiol* 1970 *219* 774—778
GORESKY C A W H ZIEGLER and G G BACH Capillary exchange modeling Barrier limited and flow limited distribution *Circulat Res* 1970 *27* 739—764
LANDIS E M and J R PAPPENHEIMER Exchange of substances through the capillary walls. *Handbook of Physiology* section 2 Circulation pp 961—1034 1963 Williams and Wilkins C Baltimore Md
LAURENT T C The structure and function of the intercellular polysaccharides in connective tissue Alfred Benzon Symposium II Capillary permeability Edit C Crone and N A Lassen pp 261—277 1970 Munksgaard Publ Copenhagen
LEFEVRE P G and A A PETERS Evidence of mediated transfer of mono-saccharides from blood to brain in rodents *J Neurochem* 1966 *13* 35—46
LEVITT D G Evaluation of the early extraction method of determining capillary permeability by theoretical capillary and organ models *Circulat Res* 1970 *27* 81—95
LONGSWORTH L G Diffusion measurements at 1 C of aqueus solutions of amino acids, peptides and sugars *J Amer chem Soc* 1952 *74* 4155—4159
PAPPENHEIMER J R E M RENKIN and L M BORRERO Filtration diffusion and molecular sieving through peripheral capillary membranes A contribution to the pore theory of capillary permeability *Amer J Physiol* 1951 *167* 13—46
PARTINGTON J R An advanced treatise on physical chemistry Vol 2 The properties of liquids Longmans Green & Co London 1951
RENKIN E Transport of potassium 42 from blood to tissue in isolated mammalian skeletal muscle *Amer J Physiol* 1959 *197* 1205—1210
SEN A K and W F WIDDAS Determination of the temperature and pH dependence of glucose transfer across the human erythrocyte membrane measure by glucose exit *J Physiol* (Lond) 1962 *160* 392—403
AP JENSEN J and N A LASSEN Restricted diffusion in skeletal muscle capillaries in man *Amer J Physiol* 1971 *220* 371—376

Abstracts from Meeting of the Scandinavian Physiological Society in Uppsala 24—25 November 1972

DEMONSTRATIONS

D 1

A Piezoelectric Driver for Glass Microelectrodes

By W Grampp and L Sjölin *Department of Physiology and Medical Physics University of Lund Sweden*

The impalement of nerve cells surrounded by tissues like connective tissue may be complicated because of the tendency of these to strongly adhere to the penetrating electrode It was felt that partly this disadvantage could be overcome by mechanically agitating the electrode tip such that it would free itself from any adhering structures thereby facilitating the advancement of the electrode through the extraneural tissue

For mechanical agitation of the microelectrode a piezoelectric driver was fitted to the electrometer amplifier probe (Fig 1) This driver is operated by means of two piezoelectric crystals (Philips PXE5) which mechanically are connected in a serial arrangement The crystals are mounted into a resonance cavity of dimensions such as to ensure a maximum flow of energy towards the electrode end of the probe The crystals are driven by electric pulses 75—150 V high and about 5 μs long which cause the driver to elongate by 0 05—0 1 μm in 2 μs.

The driving mode of the crystals has to be adjusted with respect to the electrode dimensions and the tissue to be penetrated In practical work with the abdominal stretch receptor neurons of lobster which are surrounded by a thick layer of tough connective tissue using microelectrodes of medium length and relatively blunt tip (resistances varying between 10 and 18 Mohm), it proved advantageous to drive the crystals at frequencies of 20—40 kHz for periods of 1—2 ms at a repetition rate of 2—5/s With this driving mode the electrode oscillates not only in a longitudinal

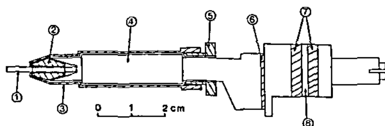


Fig 1 Vibrating microelectrode holder consisting of electrometer amplifier probe *4* and piezoelectric driver with 2 crystals *7* joined by layer of epoxy resin *8* The probe and driver compartments are fitted together by a block of fibre reinforced Bakelite *6* For attachment of the electrode *1* screw *5* pulls probe case *3* over chuck *2*

but also to some degree in a transverse direction As a result hereof the electrode performs a dissecting motion as manifested by the recurrent elimination of tip potentials arising as the electrode pushes against obstructing tissue structures

The use of the piezoelectric driver has increased the number of successful impalements of the lobster stretch receptor neurones, especially of the fast adapting neurone which previously was difficult to impale probably because of a special arrangement of its surrounding connective tissue

D 2

Measurement of Interstitial Fluid Colloid Osmotic Pressure

By K. Aukland and H M Johnsen *From the Institute of Physiology University of Bergen Norway*

As shown by Aukland and Fadnes (1972) interstitial fluid from subcutaneous tissue in rats can be collected by nylon wicks Protein concentrations of the collected fluid suggested an interstitial colloid osmotic pressure of at least 8 mm Hg

In an attempt to obtain direct measurements of colloid osmotic pressure we have developed techniques for removing native fluid from the wick and an osmometer for small volumes The colloid osmometer is a modification of that described by Tybjaerg Hansen (1961) The membrane clamping unit is made of transparent acryl plastics which is mounted directly on a Hewlett Packard pressure transducer without extra connections thus avoiding leakage problems and reducing reference fluid volume to 0 15 ml The exposed membrane area has a diameter of 3 mm and allows measurements on samples down to 4—5 μl The present experiments were made with Amicon UM 10 membranes

Interstitial fluid was collected by implanting 0 5 mm thick nylon wicks subcutaneously in rats for 1 h as described by Aukland and Fadnes (1972) For sampling fluid from the wick without evaporation the following procedure was adopted A funnel with a central opening of 1 mm diameter was placed about 1 cm above the bottom of a conical centrifuge tube filled with mineral oil (density 0 88) Immediately after removal from the rat, the wicks were transferred to the tube After centrifugation for 15 min at 3000 RPM a part of the wick fluid was found at the bottom of the tube and could be pipetted off with glass capillary and transferred to the osmometer

Preliminary measurements on wick fluid from 10 rats showed a mean colloid osmotic pressure of 11 mm Hg with a range of 9—14 mm Hg Serum colloid osmotic pressure averaged 21 (19—25) mm Hg

References

Aukland K and H O Fadnes *Acta physiol scand* 1972 *84* 26A
Tybjaerg Hansen A *Acta physiol scand* 1961 *53* 197—213

D 3

Equipment for Use in Angiographic Examinations of Animals during Diving

By A S Blix R Hol and O Iversen *Institute of Zoophysiology University of Oslo Blindern Oslo 3 Norway*

It has for long been known that submersion causes great alterations in the cardiovascular system of diving animals (Andersen 1966) Only recently however by the development of angiographic techniques has it been possible to visualize how, and to what extent these phenomena are developed

Preliminary studies by use of the above mentioned technique have in fact been carried out on head immersed animals (Aakhus and Johansen 1963 Bron *et al* 1966 and Elsner *et al* 1971) Although this condition is known to initiate the diving reflex it seems obvious that the methods used so far are inferior to one involving a fully submerged animal This condition may be obtained by the setup indicated below (Fig 1) where the animal (a seal) is enclosed in a plexiglass tube shown in cross section This tube should have a slightly greater diameter than the animal ensuring a minimum quantity of water, and preventing the animal to move While at surface position the tube should therefore be aerated Dividing on the other hand may be performed by filling the tube with water from the hydraulically operated reservoir

By this setup which may be incorporated into any modern X ray equipment any diving animal may be examined for periods only limited by the diving capacity of the animal itself

References

Andersen H T *Physiol Rev* 1966 *46* 212—243
Aakhus T and K Johansen *Acta physiol scand* 1964 62 10—17
Bron K M H V Murdaugh J E Millen R Lenthall, P Raskin and E D Robin *Science* 1966 *152* 540—543
Elsner R W N Hanafee and D D Hammond *Am J Physiol* 1971 220 1155—1157

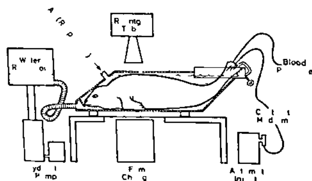


Fig 1 Schematic drawing of the equipment for use in angiographic studies of animals during diving The animal (a seal) is enclosed in a perspex tube (cross section centre) after beeing inserted through a rectangular hole at the dorso-posterior part, through which simple surgery (i e cannulations) may be performed Diving is simulated by filling the tube with water from the hydraulically operated reservoir (left)

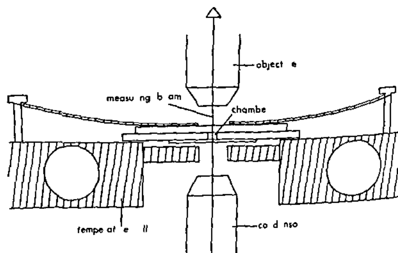
D 4

A Microspectrophotometric Method for Oxygen Consumption Studies A Comparative Evaluation

By H Herlitz and R Hultborn *Department of Physiology and Institute of Neurobiology, University of Goteborg Sweden*

Frequently used techniques for measuring oxygen consumption of small tissue fragments and isolated cells are micromodifications of manometric methods, i e the standard diver and the micro-diver techniques (e g Hamberger et al 1971) A new spectrophotometric technique for ultra microdeterminations of oxygen consumption (10^{-6} µl O /h) using hemoglobin as indicator of oxygen tension and donor of oxygen has recently been developed (Hultborn 1972)

In this study a semimicro-modification (comparable to the standard diver technique) of the spectrophotometric procedure was developed and evaluated with the standard diver technique as reference Small samples (dry weight 5—50 µg) of corpora lutea from 44 day old rats were dissected out, and respiration was determined in absence and presence of succinate (25 mM) as substrate In the spectrophotometric method fragments were placed in Tris HCl buffer to which was added a purified solution of hemoglobin to give a concentration of about 10^{-4} M The incubate was placed in a glass chamber 1 mm in diameter and 1 mm deep (Fig 1) The whole preparation was placed on a temperature cell keeping 37° C The change of absorbance of the hemoglobin solution at a certain wave length was measured in a Zeiss Microscope Photometer, and an absorbance shift curve was obtained from which the oxygen consumption was calculated The oxygen consumption of corpus luteum fragments was also determined using the standard diver technique (Hellerstrom 1967)



With the spectrophotometric method the oxygen uptake in presence of 25 mM succinate was 8 61 ± 0 72 µl O NTP/mg dry weight/h (11 determinations) and the endogenous respiration was 1 47 ± 0 12 µl O NTP/mg dry weight/h (n = 10) No significant differences under corresponding experimental conditions were found with the standard diver technique and thus the newly developed spectrophotometric technique combines easy handling with the same degree of precision as the hitherto mainly used manometric procedures

References

HAMBERGER L A HAMBERGER and H HERLITZ *Acta Endocrin* 1971 Suppl *153* 41—61
HELLERSTROM C *Endocrinology* 1967 *81* 105—112
HULTBORN R *Analytical Biochemistry* 1972 *47* 442—450

D 5

The Effect of Hemodilution and Hemoconcentration on Hepatic Plasma Flow and ICG Extraction

By N KRARUP *Institute of Physiology University of Aarhus 8000 Aarhus C Denmark*

The kinetics of hepatic dye elimination have not been definitely established yet complex models for the bromsulphalein elimination have been proposed (Winkler and Gram 1965) Whatever the correct kinetics may be it will be expected that a decrease in hepatic plasma flow must be accompanied by an increase in the hepatic dye extraction and an increase in plasma flow by a decrease in extraction ratio

The relationship between estimated hepatic plasma flow and hepatic extraction of Indocyanine Green (ICG) was studied in 12 chloralose anesthetized cats in which ICG was infused continuously at a rate of 5 µg/kg/min The cats were divided into 2 groups depending on the initial hematocrite After a control period of about 60 ml blood was withdrawn In 6 cats with a normal hematocrite (34 ± 1 6) the blood withdrawn was replaced by Macrodex (MW 70 000) and in 6 with a low hematocrite (22 ± 1 9) an erytrocyte suspension obtained from compatible donor cats was used as blood replacement

From Table I it appears that replacement with Macrodex causes an increase in hepatic plasma flow but has no effect on ICG extraction Blood replacement with erythrocytes causes a decrease in plasma flow and as expected this is accompanied by an increase in ICG extraction To further evaluate the effects the permeability surface product PS (Crone 1963) was calculated It appears that Macrodex increased the PS significantly whereas the erytrocyte suspension caused only a slight decrease in PS In the control period no significant difference is seen between cats in the two groups with different hematocrites Neither blood replacement with Macrodex nor with erytrocytes changed the splanchnic elimination of ethanol and consumption of oxygen which were determined in 4 of the experiments (2 in each group) It thus appears that a change in the hematocrite *per se* within the present range has only a slight effect on ICG elimination whereas hemodilution due to

exchange of blood with Macrodex seems to increase the liver's ability to eliminate the dye. An increase in the number of perfused sinusoids after Macrodex might explain the results. This possibility does not correspond, however, with the finding of an unaltered ethanol elimination. Metabolic effects of Macrodex may be excluded as the splanchnic oxygen consumption remained unaltered. Probably Macrodex changes the intrasinusoidal flow pattern in a way which permits more of the dye to come in contact with dye eliminating sites.

TABLE I. Estimated hepatic plasma flow (EHPF), ICG extraction ratio (E) and permeability surface product (PS = EHPF ln $\frac{1}{1-E}$) before (A) and after (B) replacement of 60 ml blood with Macrodex (group I) and erytrocytes (group II)
*** p < 0.001 ** p < 0.01 ns p > 0.10

	Number of experiments	Plasmafraction 1-hematocrite per cent		EHPF ml/kg/min		E per cent		PS ml/kg/min	
		A	B	A	B	A	B	A	B
I	6	66 ± 1.6	85 ± 1.3***	34 ± 4	49 ± 4***	20 ± 2	21 ± 2ns	7.2 ± 0.9	11.0 ± 1.1***
II	6	78 ± 1.9	56 ± 2.0***	35 ± 5	20 ± 3 **	20 ± 3	28 ± 4	7.0 ± 0.4**	6.1 ± 0.4*

References

CRONE, C. Acta physiol. scand. 1963, 58, 292—305.
WINKLER, K. and C. GRAM. Acta med. scand. 1965, 178, 439—452.

D 6

Large Osmolality Variations and Gastric Mucosal Ion Permeability and Surface Ultrastructure

By B. FRENNING. *Institute of Physiology and Medical Biophysics, Biomedical Center, University of Uppsala, Sweden.*

Hyperosmotic NaCl solutions were instilled into ligated unstimulated whole stomach pouches in cats for 30 min. On instillation of up to 620 mM NaCl the net fluxes of NaCl out of the stomachs were roughly proportional to the mean concentration difference between the gastric lumen and the blood. When more concentrated NaCl was instilled the NaCl absorption was greater than expected from this proportionality indicating that the gastric mucosal ion permeability increased on instillation of such hyperosmotic solutions. On repeated instillations of 170 mM HCl performed after an instillation of 620 mM NaCl the net fluxes of sodium ions and water into the stomach were increased over the control level and there was a net influx of chloride ions instead of a net efflux. The surface ultrastructure of gastric mucosa exposed to 620 mM NaCl as seen in the scanning electron microscope (SEM) was essentially normal supporting the view that NaCl and water probably moved into the stomach as an effect of secretion (Frenning 1972). When 170 mM HCl was instilled into stomachs previously exposed to 930 or 1550 mM NaCl the net effluxes of hydrogen ions were also increased over the control levels, a result believed to be due to an increased gastric mucosal ion permeability. On gastric mucosa from

stomachs exposed to 1550 mM NaCl and then fixed either in ordinary fixation solutions or in fixation solutions made hyperosmotic by adding NaCl intercellularly located round openings or craters were observed in the SEM On mucosa from stomachs exposed first to 1550 mM NaCl then to 170 mM HCl and finally fixed swollen and disrupted cells were seen indicating that these events occurred post hyperosmotically *in vivo* These results suggest that the increase absorption of NaCl during instillation of very hyperosmotic solutions and the increased net efflux of hydrogen ions observed during subsequent HCl instillations might mainly take place extracellularly

Reference

FRENNING B Upsala J Med Sci 1977 Suppl 13

D 7

Ionic Currents at Resting Potential in the Myelinated Nerve Fibre of Xenopus laevis

By P ARHEM and B FRANKENHAEUSER *The Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden*

One of the main pieces of evidence for regarding the resting potential as at least partly due to the potassium concentration cell is that at high external potassium concentrations the membrane behaves like a potassium electrode (Hodgkin 1964) It is well known that the potassium permeability of the nerve membrane depends on the membrane potential The behaviour like a potassium electrode might therefore apply to a fibre with low membrane potential only The present investigation was carried out in order to obtain independent measurements of the sodium potassium and chloride currents on a fibre at resting potential and thus to obtain information about the permeability conditions of the resting fibre

Potential clamp experiments were performed on myelinated fibres from *Xenopus laevis* The sodium potassium and chloride currents were calculated from measurements of the currents caused by changes of the external concentrations Some assumptions for these calculations had to be made We assumed that the chance that any individual ion will cross the membrane in a specific interval of time is independent of other ions which are present (the independence principle Hodgkin and Huxley 1952) These calculations indicated that the sum of the potassium sodium and chloride currents at the resting potential deviated from zero The consequence is either (a) the independence principle does not apply to some of these currents at rest or (b) some current (possibly caused by a metabolic electrogenic ion pump) was not measured with the present technique Metabolic inhibitors (ouabain and DNP) were therefore tested These did not change the membrane current This finding does not however exclude an electrogenic pump resistant to these inhibitors

An electrogenic pump might be unaffected by the electric driving force In this case the membrane slope conductance would be unaffected by the pump Therefore calculations of the slope conductances were made from the above described measurements using the constant field equation The calculated conductance was smaller than the conductance measured with small potential steps The conclusion from this is either (a) the constant field equation does not apply to one or all of the ionic currents or (b) the membrane has some conductance in addition to the sodium, potassium and chloride conductance

It is concluded that resting potential is not satisfactorily expressed as a potassium electrode with a small sodium error'

References

HODGKIN A L *The Conduction of the Nervous Impulse* Liverpool Univ Press 1964 p 31
HODGKIN A L and A F HUXLEY *J Physiol* (Lond) 1952 *116* 449—472

D 8

Intravenously Administered Phenylalanine and Tyrosine as Precursors of Brain Catecholamines, Effect of Chlorpromazine

By H NYBÄCK G SEDVALL, B SJOQUIST and F A WIESEL *Dept of Pharmacology Karolinska Institutet Stockholm Sweden*

The accumulation of labelled dopamine and noradrenaline in brain of mice following i v infusion of ^{14}C phenylalanine and ^{14}C tyrosine was determined Significant labelling of both amines was obtained following both precursors After administration of ^{14}C phenylalanine specific activity of ^{14}C noradrenaline exceeded that of ^{14}C dopamine Following ^{14}C tyrosine the specific activity of ^{14}C dopamine was twice that of ^{14}C noradrenaline Following chlorpromazine treatment (10 mg/kg i p) ^{14}C dopamine formation from ^{14}C phenylalanine increased about 5 fold where is dopamine synthesis from ^{14}C tyrosine increased 2 5 fold No significant effect of chlorpromazine was found on noradrenaline synthesis from either precursor The data demonstrate that chlorpromazine markedly stimulates dopamine synthesis from both ^{14}C phenylalanine and ^{14}C tyrosine From a quantitative standpoint phenylalanine seems to be a minor precursor for dopamine synthesis during resting conditions During increased demands on catecholamine synthesis as induced by chlorpromazine relatively more phenylalanine molecules are introduced directly into the catecholamine pathway

D 9

Drug Effects on Blood Pressure and Heart Rate in a Free Swimming Fish

By S S HELGASON and S NILSSON *Department of Zoophysiology University of Goteborg Sweden*

In teleost fish the branchial and systemic circulation are coupled in series making one single circuit After leaving the heart the blood flows through the ventral aorta

into the gills is recollected in the dorsal aorta and enters the systemic circulation Baro- and chemoreceptors are thought to be situated in the pseudobranchs, the false gills and send their afferent fibres in the glossopharyngeal nerve (Laurent 1967) Autonomic innervation of the heart takes place by cholinergic inhibitory and adrenergic excitatory fibres both running in the vagus nerve (Gannon and Burnstock 1969) The innervation of the systemic blood vessels may be cholinergic and adrenergic vasoconstrictor (Kirby and Burnstock 1969) while the branchial vessels are dilated by adrenaline and constricted by acetylcholine (Östlund and Fange 1962)

The present study was carried out on unanesthetized free swimming cods *Gadus morhua* Pre branchial (ventral aortic), post branchial (dorsal aortic) blood pressure and heart rate were registered simultaneously and continuously by catheters implanted in the ventral aorta and the celiac artery Drugs were injected into the animal by a catheter in the spermatic (ovaric) vein Adrenaline caused marked increase in pre and post branchial blood pressure and pulse pressure but decreased the heart rate Similar effects were seen with noradrenaline The effect on the heart rate could be abolished by atropine or scopolamine or by bilateral vagotomy This indicates a vasomotor reflex with efferent cardiodepressor cholinergic fibres in the vagus Isoprenaline produced a decrease in blood pressure and a strong increase in pulse pressure but only a slight increase if any in heart rate The alpha receptor blocking agent yohimbine produced a decreased pre and post branchial blood pressure which may indicate removal of an adrenergic tonus either nervous or hormonal The effect of adrenaline is reversed after yohimbine being then isoprenaline like Propranolol decreases heart rate and post branchial blood pressure while the pre branchial blood pressure is slightly increased This could be due to the removal of an adrenergic excitatory tonus on the heart and a dilatatory adrenergic tonus on the gill vessels The adrenergic tonus can be nervous or due to circulating catecholamines Atropine and scopolamine occasionally increased the heart rate slightly which may indicate the existence of a normally active vagal cholinergic depressor tonus on the heart

It may be suggested that the teleost circulatory system is regulated by adrenergic fibres and/or circulating catecholamines producing increased heart rate increased systemic vascular resistance and decreased branchial vascular resistance Inotropic regulation of the heart may play an important role in regulating cardiac output Cholinergic fibres running in the vagus may have some tonic depressing influence on the heart and are responsible for the efferent part of the cardio depressor reflex induced by increased blood pressure

References

GANNON B and G BURNSTOCK *Comp Biochem Physiol* 1969 *29* 765—773

KIRBY S and G BURNSTOCK *Comp Biochem Physiol* 1969 *28* 307—320

LAURENT P *C R Acad Sc Paris* 1967 *264* 1879

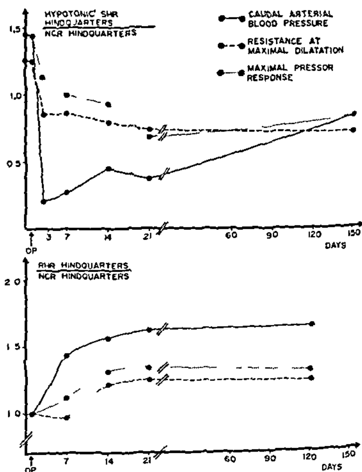
ÖSTLUND S and R FANGE *Comp Biochem Physiol* 1962 *5* 307—309

D 10

Time Course and Extent of Structural Adaptation of the Resistance Vessels in Renal Hypertensive Rats (RHR) as Compared with Spontaneously Hypertensive Rats (SHR)

By B Folkow, M Hallbäck, Y Lundgren and L Weiss *Department of Physiology, University of Goteborg, Sweden*

Earlier studies (see Folkow 1971) indicate that the resistance vessels both in essential hypertension in man and in SHR, adapt structurally to the raised pressure with an increased media thickness encroaching upon the lumen In order to study the extent and exact time course of this structural vascular adaptation and its regression renal hypertension was induced in ordinary Wistar rats by clipping one renal artery while SHR hindquarters were made hypotensive by low aortic obstruction The development of the pressure changes were followed by means of tail blood pressure



recordings and that of the structural vascular changes by estimation of their hemodynamic influence For this purpose the respective hindquarters were at different intervals after the operations perfused in parallel with normotensive control rats (NCR), as earlier described (Folkow *et al* 1970)

The SHR hypotensive resistance vessels showed clear signs of structural regression already 3—7 days after operation while those of the renal hypertensive rats (RHR) showed changes in the opposite direction within 7—14 days In both situations the respective structural adaptations appeared to be largely completed after 3 weeks (Fig 1) This was mainly judged by the changes in flow resistance at complete vasodilatation (reflecting the structurally determined inner radius) and in maximal pressor response (reflecting the contractile strength of the resistance vessels *i e* their relative media mass) —Further when the extent of these alterations in vascular design were related to the respective levels of blood pressure the SHR vessels appeared to be somewhat more affected than the RHR ones This might reflect a genetically linked quantitative difference between SHR and ordinary Wistar rats concerning their tendency to adapt structurally to a given pressure load

References

FOLKOW B *Clin Sci* 1971 *41* 1—12
FOLKOW B M HALLBÄCK, Y LUNDGREN and L WEISS *Acta Physiol Scand* 1970 *80* 93—106

D 11

Blood Flow and Capillary Filtration Capacities in Salivary and Pancreatic Glands as Compared with Skeletal Muscle

By E ELIASSEN B FOLKOW and S HILTON *Department of Physiology University of Goteborg Sweden*

Blood (or plasma substitute) flow capillary exchange capacities in vascular beds with fenestrated and continuous capillary walls have been compared in experiments on the isolated pancreas or submaxillary salivary glands (representing the former) or skeletal muscle (representing the latter)

Using cats dogs and piglets the vascular beds mentioned were so isolated as to allow continuous recordings of arterial inflow and venous outflow pressures (P_A P_V) blood (or plasma substitute) flow and organ weight (or volume) This made it possible to estimate capillary filtration coefficient (CFC) for evaluation of differences in capillary surface area—permeability (PS) values mean capillary pressure (P_C) was estimated by the Pappenheimer method Measurements were performed during both resting vascular tone and maximal vasodilatation produced by papaverine or bradykinin the latter being a physiological vasodilator and also a permeability increasing factor in the glands The results so far are summarized as range of values in Table I to give a general impression of the differences in vascular dimensions

TABLE I

	Blood flow ml/min × 100 g at $P_a - P$ = 100 mm Hg		Mean cap pressure mm Hg at $P_a - P_v$ = 100 mm Hg		Cap filtr coeff ml/min × mm Hg × 100 g		
	rest	max dil by papaverine	rest	max dil by papaverine	rest	max dil by papaverine	max dil. by bradykinin
Skeletal muscle	6 – 10	45 – 60	12 – 14	20 – 25	0 01 – 0 015	0 04 – 0 05	0 07 – 0 1
Pancreas	25 – 40	300 – 400	9 – 11	> 20	0 1 – 0 3	0 7 – 0 8	1 3 – 1 6
Submaxill gland	30 – 50	400 – 600	9 – 11	> 40	0 1 – 0 3	0 8 – 1 0	1 5 – 2 0

The table illustrates the enormous capacity of capillary filtration transfer in salivary glands during maximal functional hyperemia (60—80 ml/min × 100 g) compared with skeletal muscle (0 15—0 20 ml/min × 100 g) This is partly due to the far higher PS values, partly to the much higher P_C increase in the gland, which is necessary in organs that may actively secrete almost their own weight/min Similar to the glomeruli which provide the renal tubular cells with an abundant ultrafiltrate for subsequent active and selective transfer the salivary gland capillary bed provides the gland cells with a filtrate used for saliva formation and secretion but its maximal filtration capacity per unit organ weight under the influence of bradykinin actually seems to be nearly twice that of the kidney

D 12

Changes in Crural Blood Flow Caused by Oxygen Breathing at 1 Atmosphere

By M HANSEN and J MADSEN *Medicinsk fysiologisk institut C University of Copenhagen Denmark*

Change of breathing medium from air to oxygen at 1 atm causes an 11 per cent reduction in calf (Reich *et al* 1970) and forearm blood flow (Bird and Telfer 1966) of resting men examined by plethysmography Bird and Telfer noticed that the reduction in blood flow persisted for some time after cessation of oxygen administration Similar observations were made on dog hind limbs No figures were given for the duration of this after effect The present experiments were carried out to examine the blood flow changes in a calf segment at the beginning and at the end of a 20 min period of oxygen breathing

One experiment was made on each of 9 healthy male volunteers The subject laid immobile on his back with the calf placed in a water plethysmograph 0 cm above the level of the couch The arrangement and use of the plethysmograph were essentially as described by Paulev ([illegible] rest of the experimental setup has been described by Hansen and Mad[illegible] Most readings were taken with 1—?

min intervals For each 5 min period the average value was used for further calculations

The average flow rate during air breathing before the oxygen period was 1 9 ml 100 ml^{-1} min^{-1} (S D 0 46) Calf blood flow was reduced during oxygen breathing For each experiment the change was calculated as a fraction of the initial blood flow The average reduction was −6 per cent in the 0—5 min period of oxygen breathing (0 1 > P > 0 05) and reached its maximum value of −12 per cent (S E 2 6) in the 5—10 min period (P < 0 002) Upon cessation of oxygen breathing the flow increased This increase became significant in the 5—10 min period after breathing gas change (P < 0 001) In the 0—5 min post oxygen period he flow rate averaged 97 per cent (S E 4 2) of the pre oxygen control flow in the 5—10 min period 106 per cent (S E 2 9) The average values were statistically indistinguishable from the pre oxygen control values both in the 0—5 and in the 5—10 min periods after resumption of air breathing (P > 0 05)

References

BIRD A D and A B M TELFER *Surg Gynec Obstet* 1966 *123* 260—268
HANSEN M and J MADSEN *Scand J clin Lab Invest* 1972 *30* 43—48
PAULEV P E *Acta physiol scand* 1969 *Suppl 324* 24—33
REICH T J TUCKMANN N E NAFTCHI and J H JACOBSEN *J appl Physiol* 1970 *28* 275—278

D 13

Determination of Adipose Tissue Blood Flow in the Intact Rat by ^{133}Xe Elimination

By J MADSEN A MALCHOW MOLLER and S WALDORFF *Medicinsk fysiologisk institut C København Denmark*

The purpose of the present experiments was to develop a method for continuous registration of adipose tissue blood flow (ATBF) in intact small animals

Rats were labelled with ^{133}Xe and the elimination followed by external scintillation counting in 3 ways *A* Unanesthetized rats were placed for several hours in a closed system containing ^{133}Xe After labelling they were enclosed in an immobilizing cage while scintillation counting was carried out over the abdomen *B* Anesthetized rats were labelled by breathing through a tracheal cannula from a closed circuit system containing ^{133}Xe A collimated scintillation detector was placed over the lower abdomen of the animal to monitor the elimination *C* ^{133}Xe was injected i p to rats Counting was performed as described for A or B

The elimination curves were made up of 1 to 3 monoexponential components depending on the method of labelling and the region of the body seen by the detector Analysis of tissues from animals killed at varying times during the elimination showed that one of these components reflects ^{133}Xe elimination from adipose tissue From 27 expts of type B ATBF of fed rats was calculated to 11 5 ml 100 g^{-1} min^{-1} (S D 3 9) using a tissue blood partition coefficient for Xe of 10 1 ml g^{-1} Results obtained by methods A and C did not differ significantly from this average Our find

ings are in accordance with figures obtained by other methods (Herd *et al* 1968 Mayerle and Havel 1969)

Method A is complicated by the occurrence of an elimination curve component slower than that of fat ($T_{1/2}$ 6—10 h) It was shown to be caused by [133]Xe retention in the fur Method B nearly always yielded monoexponential elimination curves reflecting [133]Xe elimination from fat in the 2—6 h period after beginning of elimination After 6 h a decrease in elimination rate was seen in some experiments It could be attributed to the slower washout of [133]Xe from intestinal content Work on method C is in progress Apparently abdominal fat is labelled as in method B The method s main advantage is the possibility of experiments on unanesthetized animals

References

HERD J A H M GOODMAN and S A GROSE *Amer J Physiol* 1968 *214* 263—268
MAYERLE J A and R J HAVEL *J appl Physiol* 1969 *26* 513—516

D 14

Exercise Ventilation and Metabolism during Hyperthermia in Man

By E STRANGE PETERSEN and H VEJBY CHRISTENSEN *Institute of Physiology University of Aarhus Denmark*

At rest it is well established that minute ventilation is increased during hyperthermia (*cf* Cunningham and O Riordan 1957) During exercise however the importance of a temperature stimulus to breathing is less clear (Dejours *et al* 1958 Whipp and Wasserman 1970) The purpose of this study was to evaluate the possible significance of temperature as a respiratory drive during exercise

4 healthy subjects aged 19—23 entered the study At normal and elevated (+1 4° C) rectal temperature ventilation oxygen uptake carbon dioxide elimination, heart rate and concentrations of lactate and pyruvate in arterialized capillary blood were measured during steady state bicycle ergometer exercise of different intensities (300—1000 kpm/min) Transient changes in ventilation were studied at the onset of work breath by breath The experiments at high body temperature were performed in an environmental chamber (Andersen and Lundqvist 1970) with air temperature about 44° C and high relative humidity

One consistent finding in the ON transient in hyperthermia was a marked overshoot in respiratory frequency In the steady state at equal loads minute ventilation was the same although frequency was higher and tidal volume lower at elevated than at normal body temperature Oxygen uptake was significantly lower ($P <$ 0 001 paired comparisons) and the ventilatory equivalent therefore higher in hyperthermia than in normothermia The blood lactate concentration was higher at rest and at all work loads indicating an increased anerobic energy yield in hyperthermia this could be explained by relative anerobiosis in the working muscles due to increased cutaneous blood flow with concomitant decreased muscle blood flow In a temperature interval from 35 5—37 8° C Whipp and Wasserman (1970) found the

ventilatory equivalent for CO constant during work of differing intensity In our experiments the ventilatory equivalent was increased at body temperatures above 38° C implying the existance of some threshold in this region The increase in lactate concentration in hyperthermia implies a comparably decreased HCO_3 concentration No major changes in PaCO are expected because alveolar ventilation is decreased and CO output also lowered in hyperthermia An increased (H) stimulus might therefore constitute the additional drive which causes the increment in the ventilatory equivalent

Temperature is thus hardly an independent respiratory stimulus during exercise in man although an effect on the rate determining mechanisms (*cf* Euler *et al* 1970) appears to be well documented This might be the remnants of a panting mechanism in man

References

ANDERSEN I and G R LUNDQVIST *Int J Biometeor* 1970 *14* 402—405
CUNNINGHAM D J C and J L H O RIORDAN *Quart J exp Physiol* 1957 *42* 329—345
DEJOURS P A TEILLAC F GIRARD and A LACAISSE *Rev Franç Études Clin Biol* 1958 *3* 755—761
EULER C VON F HERRERO and I WEXLER *Respir Physiol* 1970 *10* 93—108
WHIPP B B and K WASSERMAN *Respir Physiol* 1970 *8* 354—360

D 15

Changes in the Lactate Dehydrogenase Isoenzyme Pattern of Human Brains Following Lifelong Exposure to Hypoxia

By P HELLUNG LARSEN M A JENSEN and S C SORENSEN *Institute of Medical Physiology Dept A and Institute of Biochemistry Dept B University of Copenhagen Denmark*

The pattern of lactate dehydrogenase (LDH) isoenzymes in tissues might change when Po is altered *in vivo* or *in vitro* (Thorling and Jensen 1966 Hellung Larsen and Andersen 1969) It has been suggested that a predominance of H subunits in a tissue is correlated to aerobic metabolism and that a predominance of M subunits is correlated to anerobic metabolism (Pfleiderer and Wachsmuth 1961) The increase in the relative amount of M subunits which is observed when Po is lowered is compatible with this hypothesis Thus accordingly the LDH pattern in a tissue reflects the metabolic state aerobic versus anerobic metabolism

We wanted to examine whether the glucose metabolism in the brains of people who live at high altitude is more anerobic than in people who live at sea level because an increased anerobic metabolism in the brains of people living at high altitude might explain ventilatory acclimatization to chronic hypoxia (Sorensen 1971)

We therefore examined the LDH isoenzyme pattern in brains from people who had been exposed to hypoxia at high altitude for their entire life We have examined the LDH isoenzyme in grey matter from the cerebral cortex *in white matter from* corpus callosum and in cerebellar cortex in five human brains obtained at autopsy in La Paz Bolivia (altitude 3800 m) and in six brains obtained at autopsy from a

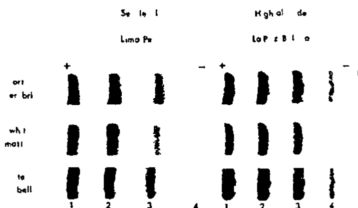


Fig 1 LDH isoenzyme patterns of different parts of the brain of human living at sea level or at high altitude

genetically similar group at sea level in Lima Peru The LDH isoenzyme distribution was determined in clear supernatants from tissue samples homogenized in saline (tissue/saline = 1/3 W/V) The isoenzymes were separated by agarose gelelectrophoresis (8) The electrophoresis was run for 45—60 min LDH was subsequently visualized by the nitroblue tetrazolium staining technique (Hellung Larsen 1968)

Fig 1 shows the LDH isoenzyme pattern in brains from sea level and from high altitude There was some variation in the pattern at both altitudes but those shown are representative The difference between the 2 groups is best seen as the lower ratio of LDH 2/LDH 3 at high altitude compared with sea level indicating a higher relative concentration of M subunits at high altitude The difference between sea level and high altitude is most pronounced in white matter

The results support our suggestion that the brain produces more lactic acid at high altitude than at sea level

References

Hellung-Larsen P *Acta chem scand* 1968 22 355—358
Hellung Larsen P and V Andersen *Exptl Cell Res* 1969 *334* 185—190
Sorensen S C *Acta physiol scand* 1971 Suppl 371
Thorling E B and K Jensen *Acta path microbiol scand* 1966 *66* 426—436

D 16

Evidence against Erythropoietin Production by the Carotid Bodies

By J Fogh A J Hansen K Mollgard and S C Sorensen *Institute of Medical Physiology Dept A Institute of Anatomy Dept A and Department of Nuclear Medicine University of Copenhagen Denmark*

Recently Tramezzani *et al* (1971) presented experimental evidence which indicated that in cats, the carotid bodie produce erythropoietin or an erythropoietin like

substance during anemia. Assuming that erythropoietin production is stimulated by the same mechanism during anemia and hypoxia we examined in cats whether the carotid bodies are essential for the increase in erythropoietin production in blood during hypoxia. We compared the effect of 6 h of hypoxia (P_IO_2 = 60—70 mm Hg) on the erythropoietin concentration in serum in 5 normal cats and in 5 cats which had their carotid bodies surgically removed several weeks prior to the hypoxic exposure. The erythropoietin concentration in serum was measured with a biological assay method described by Fogh (1966). We found that the erythropoietin concentrations in serum after hypoxia were not lower after carotid body removal as compared with the concentration in serum obtained from normal cats after hypoxic exposure. Actually the erythropoietin concentrations were significantly greater in cats which had their carotid bodies removed. This, however, might be because they do not hyperventilate as much during hypoxia as the normal cats. Therefore, the arterial P_{O_2} was lower in the operated cats during hypoxia than in the normal cats. We also measured the erythropoietin concentration in homogenates from ten carotid bodies from cats. The homogenates were prepared in the way Tramezzani *et al* (1971) described but we were not able to detect any erythropoietin in these homogenates.

Erythropoietin is a glycoprotein and we therefore examined histochemically whether carotid bodies from rabbits contained PAS positive maltase resistent material. We examined carotid bodies from 2 normal rabbits and from 2 rabbits which had been exposed to hypoxia (equivalent to an altitude of 5000 m) for 7 days. There was only a weak diffuse PAS positivity and no PAS positive granules and there was no difference between the 2 groups.

Our results therefore do not support the suggestion that the carotid bodies produce erythropoietin.

References

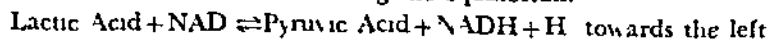
FOGH, J. *Scand J Clin Lab Invest* 1966 *18* 33—44.
TRAMEZZANI, J. H., E. MORITA and S. R. CHIOCCHIO. *Proc Nat Acad Sci* 1971 *68* 52—55.

D 17

Glycerol and the Postexercise Lactate Elimination

By C. OLSEN and E. STRANGE PETERSEN. *Institute of Physiology, University of Aarhus, Denmark*

It has been shown that rather low alcohol concentrations can inhibit the conversion of lactate to glucose in the perfused rat liver (Krebs *et al* 1969 b) and in man following infusion of ^{14}C labelled lactate (Kreisberg *et al* 1971) and severe exercise (Krebs *et al* 1969 a). The mechanism underlying this observation is probably a reduction of NAD to NADH driving the equilibrium


Lactic Acid + NAD ⇌ Pyruvic Acid + NADH + H towards the left.

As the oxidation of glycerol predominantly takes place in the liver (Larsen 1963,

and resembles that of alcohol, it might be expected that elevated glycerol concentrations in the blood likewise could affect the elimination rate of lactate following exercise in man This might even be the case with physiologically raised glycerol concentrations in the postexercise period We have consequently studied the effect of raised glycerol concentrations on the concentrations of lactate and pyruvate and the lactate/pyruvate ratio in blood in 7 young adults at rest and after exercise The procedure and methods were as described before (Olsen and Strange Petersen 1971 Olsen 1971) but in one type of experiment the subjects were given glycerol orally before work started and at intervals during the recovery period

The following observations were made *1* 10 min of exercise induced a significant rise in the glycerol concentration *2* The concentration of lactate at rest was significantly higher after than before glycerol intake *3* The lactate/pyruvate ratio was significantly higher after than before glycerol intake both at rest and after 15 min of recovery and onwards *4* The metabolic clearance of lactate after exercise was reduced by the glycerol intake to about 70 % of the control value *5* The effect of glycerol on the metabolic clearance of lactate was the same whether estimated at rest or after exercise

These observations are in accordance with the concept that glycerol metabolism influences the lactate/pyruvate ratio in the liver and thereby reduces the clearance of lactate

References

KREBS H A D J C CUNNINGHAM M STUBBS and D J A JENKINS *Israel J Med Sci* 1969 a *5* 959—962
KREBS H A R A FREEDLAND R HEMS and M STUBBS *Biochem J* 1969 b *112* 117—124
KREISBERG R A W C OWEN and A M SIEGAL *J Clin Invest* 1971 *50* 166—174
LARSEN J A *Acta physiol scand* 1963 *57* 224—234
OLSEN C *Clin Chim Acta* 1971 *33* 293—301
OLSEN C and E STRANGE PETERSEN *Acta physiol scand* 1971 *82* 10 A—11 A

D 18

Impedance Measurements on Frog Skin during Various Stages of Anoxia

By E BLOMQUIST and J SANDBLOM *Inst of Physiology and Medical Biophysics Biomedical Center University of Uppsala Sweden*

Impedance measurements on epithelial membranes have been used to characterize their dielectric properties (Teorell and Wersall 1945) as well as the ion conduction pathways (Flemstrom 1971) It has also been discovered that partial anoxia of the frog gastric mucosa causes a concomitant increase of transmembrane impedance and active sodium transport (Flemstrom 1971) We have measured the membrane potential the short circuit current and the membrane impedance of the frog skin during various stages of anoxia The impedance locus is composed of two separate dispersion regions which were found to decrease at an early stage of anoxia followed later by an increase in the impedances These changes have been interpreted in terms of

redistribution of sodium ions between intraepithelial compartments as a result of changes in active transport produced by the anoxia

References

FLEMSTROM G *Biochem biophys Acta* (Amst) 1971 2?5 3?—45
TEORELL T and R WERSALL *Acta physiol scand* 1945 10 ?43—257

D 19

Na K ATPase Isoenzymes with Different Temperature Characteristics in the Nervous System of Ectotherms

By J KOHONEN K Y H LAGERSPETZ and R TIRRI *Zoophysiological Laboratory Department of Zoology University of Turku Finland*

The temperature dependence of the Na K ATPase activity in homogenates and microsomal preparations from the brain shows a simple curvilinear course in adult rats while the relationship in young rats aged from 1 to 15 days consists of 2 activity maxima at 45—50° C and at 20—24° C respectively In young rats with a still undeveloped thermoregulation the enzyme activity is thus less sensitive to temperature than in adult rats

The Na K ATPase activity in brain homogenates from a cold water fish the roach (*Leuciscus rutilus*) shows curvilinear temperature dependence with two activity maxima (at 41° C and at 20° C respectively) while the enzyme activity in brain homogenates from a warm water fish the kissing gourami (*Helostoma temmincki*) has only one maximum at 45° C

The Na K ATPase activity of nerve cord homogenates from earthworms (*Lumbricus terrestris*) acclimated to 14° C was less sensitive to temperatures below 35° C than that of earthworms acclimated for 3 weeks to 25° C

The complex curvilinear form of the temperature dependence of the Na K ATPase activity may be explained by assuming the existence of 2 or more Na K ATPase isoenzymes with different temperature characteristics in the nervous tissue of ectothermic animals The isoenzyme with the activity maximum at lower temperatures may be induced by temporary hypothermia which occurs in young rats with an undeveloped thermoregulation and by cold acclimation in poikilothermic animals

Other evidences than those found in the present study can be adduced for the isoenzyme hypothesis Na K ATPase activity at low temperatures is less sensitive to the inhibitory action of ouabain and the K requirement for maximal activity is lowered with decreasing temperature (Ahmed and Judah 1965) These observations indicate a chemical difference in the enzymes active at high and low temperatures In addition the changes caused by temperature acclimation in the temperature dependence of the Na transport linked with Na K ATPase activity in goldfish intestinal mucosa are inhibited by the impairment of protein synthesis with puromycin (Smith 1966 1967 Smith and Morris 1966) Also the Na K ATPase activity of brain microsomes from hibernating hedgehog is less temperature sensitive than that of brain microsomes from awake hedgehogs (Bowler and D 9)

References

AHMED K and J D JUDAH *Can J Biochem* 1965 *43* 877—880
BOWLER K and C J DUNCAN *Physiol Zool* 1969 *42* 211—219
SMITH M W *Experientia* 1966 *22* 252—253
SMITH M W *Biochem J* 1967 *105* 65—71
SMITH M W and D MORRIS *Experientia* 1966 *22* 678—679

D 20

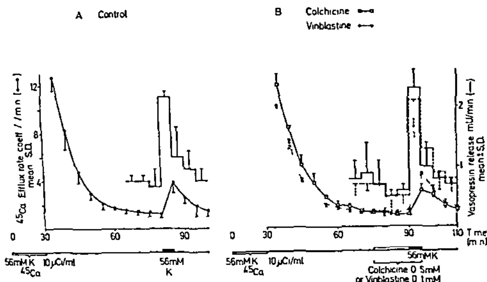
Lack of Inhibition by Colchicine and Vinblastine of Vasopressin Release and Calcium Efflux from Isolated Rat Neurohypophyses

By J T RUSSELL and N A THORN *Institute of Medical Physiology C University of Copenhagen Denmark*

Malaisse (1972) has reported that the level of calcium in the cytosol might regulate the exocytotic process of insulin release by controlling the activity of the microtubule system of the β cells This hypothesis was based on the finding that colchicine inhibited the glucose induced insulin release from isolated β cells (Lacy *et al* 1968) That colchicine and certain Vinca alkaloids disrupt the microtubular system in cells at very low concentrations (10^{-9} g/ml) has been well documented (Dustin 1963, Malawista Sato and Bensch 1968) However in the secretion mechanisms in the adenohypophysis colchicine did not have any inhibitory effect (Kraicer and Milligan 1971) Since there is some evidence that the release of vasopressin from the neurohypophysis occurs through a process of exocytosis we have tested the effect of colchicine and vinblastine on the potassium and electrically stimulated release of vasopressin and calcium efflux from isolated rat neural hemilobes

Isolated neurohypophyseal hemilobes (5 for each experiment) were incubated for the first 30 min after removal in McIlwain Rodnight medium containing 56 mM K and 10 μCi/ml ^{45}Ca (Radiochemical Centre Amersham Lot CFS 7D[2]) After this the hemilobes were transferred to a teflon chamber and a washout was carried out with 2 ml of non radioactive medium (4 8 mM K) changing every 5 min ^{45}Ca efflux into the washout medium was measured by liquid scintillation counting (Packard Tricarb Model No 3375) using a Triton X 100 emulsion counting system Vasopressin release into the medium was assayed by the blood pressure method in rats During the washout an exposure to a high K medium (56 mM) resulted in a 2 to 3 fold increase of vasopressin output (0 79 ± 0 14 to 2 20 ± 0 10) and of the rate of ^{45}Ca efflux (1 19 ± 0 32 to 3 93 ± 0 86) (Fig 1 A 3 expts) Addition of colchicine (0 5 mM) or vinblastine (0 1 mM) to the washout medium did not alter the rate of release of vasopressin or ^{45}Ca from the lobes either in resting glands or during potassium stimulation (Fig 1 B 3 expts with each drug) The electrically stimulated release was also not affected

Recently colchicine and vinblastine have been shown to inhibit acetylcholine induced catecholamine release from adrenal medulla but not the potassium induced release (*e g* Trifaro *et al* 1972) The former was thought to be an anticholinergic action rather than an effect on the microtubules The results presented here demon



strate that an increased efflux of Ca^{++} occurs during potassium stimulated release of vasopressin Further they suggest that microtubules are not involved in the hormone extrusion process

References

DUSTIN P JR *Pharmac Rev* 1963 *15* 449—480
KRAICER J and J V MILLIGAN *Endocrinology* 1971 *89* 408—412
LACY P E S L HOWELL D A YOUNG and C J FLINK *Nature* (Lond) 1968 *219* 1177—1179
MALAISSE W J *Isr J Med Sci* 1972 *8* 244—251
MALAWISTA S E H H SATO and K G BENSCH *Science* 1968 *160* 770—772
TRIFARO J M B COLLIER A LASTOWECKA and D STERN *Molec Pharmacol* 1972 *8* 264—267

D 21

Isolation of Parietal Cells from the Gastric Mucosa

By T BERGLINDH *and* K J ÖBRINK *Department of Physiology and Medical Biophysics Biomedical Center University of Uppsala Sweden*

In order to understand the mechanism behind gastric acid secretion it is necessary to work also on cellular levels Therefore attempts have been made to isolate parietal cells (*cf* Blum *et al* 1971)

A rabbit stomach was made blood free by perfusion *in situ* with cold phosphate buffered saline (PBS) through a cannula in the abdominal aorta then quickly taken out and cut open The fundic part was rinsed several times in cold oxygenated PBS after which the mucosa was stripped off minced and transferred to a balanced calcium free salt solution (BSS) containing 0 15 % (w/v) Pronase® After 15 min in 37° C most of the epithelial cells were found in the solution The mucosa was then

transferred to another BSS with 0.05 % (w/v) collagenase. After 45 min practically all the cells were digested free. Through the whole procedure care was taken to keep the cells oxygenated. The cells were transferred to cold 16 % (w/v) polyethylene glycol (PEG) and then separated on a density gradient in a gravity field of 850×g. The gradient was made from colloidal SiO_2 (Ludox®) and PEG (M.W. 4000). See Pertoft (1969). This technique makes it possible to keep the cells at constant pH and osmolality within physiological ranges.

After 50 min the cells at different isopycnic niveaus were collected. The parietal cells were concentrated at a density of 1.07—1.08 g/cm³ where they constituted approximately 95 % of the cell population. The cells were washed free from Ludox® and suspended in a Ringer solution.

The parietal cells were identified: a) in phase contrast microscope where they appeared as big (average 20 μm) dark granulated cells with a central nucleus, b) by determination of succinic dehydrogenase with nitrotetrazolium blue which gives the parietal cell a dark blue appearance as a sign of a very high mitochondrial content and b) by eosin hematoxylin and PAS staining.

Viability of the cells was controlled with the dye exclusion technique using eosin erythrosin or trypan blue. In successfull experiments a viability between 80 and 90 % of the parietal cell population was obtained.

References

BLUM, A. L., G. T. SHAH, V. D. WIEBELHAUS, F. T. BRENNAN, H. F. HELANDER, R. CEBALLOS and G. SACHS: *Gastroenterology* 1971. *61*. 189—200.

PERTOFT, H.: *Exptl. cell Res.* 1969. *57*. 338—350.

D 22

Macromolecular Permeability and Acid Secretion in Isolated Gastric Mucosa after Acetylsalicylic Acid

By G. FLEMSTRÖM and N. V. B. MARSDEN. *Department of Physiology and Medical Biophysics, Biomedical Center, University of Uppsala, Sweden*

It is well known that unionized acetylsalicylic acid (ASA) in the gastric lumen increases the mucosal ionic permeability. There is strong evidence that the latter underlies its physiological effects. Some other weak acids exert a similar effect (*cf.* Davenport 1970) which is probably due to intracellular accumulation of the conjugate base (Flemström 1971). There is evidence also for an increased cellular volume (Frenning and Öbrink 1971). Swollen mucosal cells may conceivably affect gastric permeability in 2 ways. There may be firstly increased cellular permeability and thus increased transcellular ion transport and secondly it may not be possible to accomodate the deformed swollen cells in the mucosa without changing the dimensions of the paracellular space in such a way as to increase its permeability.

In order to test and if possible characterize permeability changes resulting from exposure of the luminal side of the gastric mucosa to ASA we have studied the transmucosal migration of saccharides, *i.e.* polydisperse dextran fractions and raffinose in

isolated frog (*Rana temporaria*) gastric mucosal preparations This preparation permitted the concomitant determination of hydrogen ion secretion ionic conductance and transmucosal electrical potential difference The dextran mol wt distribution was determined by gel permeation chromatography the *in vitro* technique has been described earlier (Flemstrom 1971)

Both 5 and 10 mM ASA (pH 3 00) reduced the electrical potential difference depressed the rate of hydrogen ion secretion and increased mucosal conductance and saccharide permeability The steady flux levels after 5 and 10 mM ASA (pH 3 00) were approximately the same for the respective saccharides Normally the mucosa is impermeable to raffinose (equivalent diffusion radius = 5 6 Å) After 5 mM ASA the upper limit was about 40 Å (dextran mol wt = 30 000)

Unionized ASA (pH 3 00) in concentrations of 3 mM or lower and 10 mM ionized ASA (pH 7 12) did not significantly effect the electrical potential difference or the ionic conductance but there was a decrease of hydrogen ion secretion Permeation of raffinose was not detected with either 1 mM (pH 3 00) or 10 mM ASA (pH 7 12)

Since the saccharide fluxes were approximately the ame above 5 mM ASA and zero under 3 mM ASA the permeability increase apparently develops over a narrow critical concentration range and may be an *all or none* type of effect The conductance was however still relatively low ($<$ 6 mMho/cm) when dextran started to permeate which probably indicate considerable integrity of the mucosa A paracellular migration of dextran therefore seems more likely particularly in view of the size of the molecules penetrating It is perhaps relevant that molecules sufficiently large to evoke immune responses penetrated the ASA treated mucosa

References

DAVENPORT H W In *Progress in gastroenterology* Vol II Ed G B J Glass Grune & Stratton Inc New York 1970 pp 42—56

FLEMSTROM G *Acta physiol scand* 1971 *82* 1—16

FRENNING B and K J ÖBRINK *Scand J Gastroent* 1971 6 605—612

D 23

Can the Sympathetic Large Dense Core Nerve Vesicles be Transformed to Small Vesicles by Depletion of their Soluble Content in Vitro

By H LAGERCRANTZ *Department of Physiology Karolinska Institute Stockholm Sweden*

Chubb De Potter and De Schaepdryver (1972) Smith and Winkler (1972) and Kopin and Silberstein (1972) have suggested that the heavy large den e core vesicles in sympathetic nerves release their content by exocytosis in a similar way as the chromaffin granules After release they form light small dense core vesicles The present study indicates that catecholamine is stored in a different way in large dense core vesicles than in chromaffin granules and therefore might be released in a different way

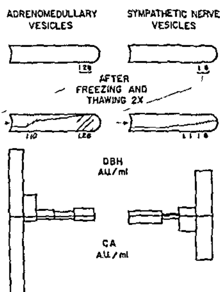


Fig 1 A fraction containing mainly sympathetic nerve vesicles isolated in a sucrose density gradient, was recentrifuged on a new density gradient after freezing and thawing twice Catecholamines (CA) and dopamine β hydroxylase (DBH) activities expressed in arbitrary units (AU) were analyzed in 4 fractions obtained from the gradient To the left is shown a similar experiment with adrenomedullary vesicles The specific weights are indicated below the gradients

Highly purified large dense core vesicles were isolated from bovine splenic nerves (see Lagercrantz 1971) and frozen and thawed twice and then centrifuged on sucrose density gradients Similar treatment of the adrenomedullary vesicles leads to a prompt release of most of the catecholamines and dopamine β hydroxylase and decrease of the density of the vesicles in analogy of what is happening *in vivo* (see Fig 1 modified after Viveros Arqueros and Kirshner 1971) However the nerve vesicles retain their density and also most of their noradrenaline and proteins (Fig 1) This *finding indicates* that the nerve vesicles may contain only an evolutionary rest of the large non diffusible catecholamine ATP soluble protein complex found in the adrenomedullary vesicles and that noradrenaline is distributed in a principally different way in the nerve vesicles This idea is further supported by 1) the occurrence of only trace amounts of chromogranin A in the soluble lysate of nerve vesicles (Bartlett Smith and Lagercrantz to be published) 2) the striking differences between intact adrenomedullary and nerve vesicles but similarities between intact nerve vesicles and adrenomedullary vesicle membranes with regard to chemical composition and noradrenaline binding capacity (Lagercrantz 1971 Helle Lagercrantz and Stjarne to be published) 3) the total exchangeability of the noradrenalin storage pool in the nerve vesicles (Klein and Lagercrantz 1971)

The present results do not support the idea that the heavy large dense core vesicles become light small dense core vesicles only by depletion of their soluble content

References

CHUBB I W W P DE POTTER and A S SCHAEPDRYVER *Experientia* 1972 *28* 293—295
KLEIN R L and H LAGERCRANTZ *Acta physiol scand* 1971 *83* 179—190
KOPIN I and S SILBERSTEIN *Pharmacol Rev* 1972 *24* 245—254
LAGERCRANTZ H *Acta physiol scand* Suppl *366* 1971
SMITH A D and H WINKLER *Handbook of Exp Pharmacol* 1972 *33* 538—61
VIVEROS O H L ARQUEROS and N KIRSHNER *Mol Pharmacol* 1971 *7* 445—454

D 24

Restricted Placement of α adrenergic Receptors within the Smooth Muscle of the Rat Portal Vein

By J A BEVAN and B LJUNG *Department of Pharmacology UCLA School of Medicine Los Angeles California 90024 and Department of Physiology University of Goteborg Sweden*

The outer longitudinal muscle layer of the rat portal vein is 4 times thicker than the inner circular muscle coat (Johansson *et al* 1970) Neuronal noradrenaline (NA) uptake is essentially confined to the plexus (Ljung and Bevan 1972) that separates the 2 layers (Johansson *et al* 1970) In view of this morphological arrangement the finding of considerable denervation supersensitivity was interpreted to indicate that only smooth muscle cells in the vicinity of nerve terminals directly respond to exogenous NA (Johansson *et al* 1970 Ljung and Bevan 1972)

This concept was tested by measuring the delay before the onset of a contractile response of the longitudinal muscle layer in veins exposed to NA either *via* their adventitial or intimal surface The mean difference between latency in onset of response to NA (10^{8} 10^{7} 10^{6} M) following exposure of portal veins on their adventitial and on their intimal surfaces was 5 7 5 0 and 3 0 s respectively Small or inconsistent differences were found in similar studies with acetylcholine (10 and 10^{6} M) and following potassium increase (×8) Dose response curves to NA applied via either the adventitial or intimal surface and the consequence of cocaine (10^{6} M) on these curves were essentially identical

The considerable difference in delay of the response to NA applied to the outside when compared to the inside application taken in conjunction with the small differences in delay to non adrenergic agonists is consistent with the idea that NA applied to the outside must diffuse through the greater thickness of the longitudinal layer before initiating a re ponse NA entering from the inside would have to traverse only 25 % of this distance before reaching the same muscle cells Apparently the non adrenergic agonists act on surface muscle cells in both instances Since the dose response curves to NA entering either *via* the adventitia or intima were the same neuronal uptake must affect the concentration of NA at its site of action equally irrespective of its direction of entry These observations support the proposition that only smooth muscle cells in the vicinity of the adrenergic nerve terminal are provided with α adrenergic receptors in the rat portal vein

References

JOHANSSON B B LJUNG T MALMFORS and L OLSON *Acta physiol scand* 19 0 Suppl *349* 17—32

LJUNG B and J A BEVAN *Circulat Res* 1972 (In P ess)

D 2ɔ

Oncotic and Hydrostatic Forces Operative over the Peritubular Capillary Membrane

By Ö Källskog and M Wolgast *Department of Physiology and Medical Biophysics Biomedical Center University of Uppsala Sweden*

It has been suggested that the driving forces operating over the peritubular capillary membrane should be a factor determining the tubular fluid reabsorption To investigate this problem the hydrostatic and oncotic forces on the 2 sides of this membrane have been investigated in a series of experiments on rats The capillary hydrostatic pressure was determined by micropuncture of vessels within the peritubular network using a servo controlled counter pressure device according to Wiederhielm *et al* (1964) The oncotic pressure was estimated from the plasma protein concentration and the filtration fraction—the latter was determined from glomerular filtration rate and renal plasma flow The interstitial pressure was measured via small (50 μm) PVC catheters put under the renal capsule into the subcapsular space which was assumed to be representative for at least superficial renal interstitium The interstitial oncotic pressure was calculated from protein concentration and the albumin globulin ratio found in this space as determined from samples withdrawn via the small subcapsular catheters The hydrostatic pressure in the capillaries amounted to 11 1 ± 1 1 mm Hg (mean ± S D) and in the interstitium to 1 3 ± 0 6 mm Hg The intravascular oncotic pressure averaged 27 7 mm Hg and the corresponding interstitial pressure 4 0 mm Hg (protein concentration 1 5 %) The net driving force will then be some 14 mm Hg In extracellular volume expansion the interstitial protein concentration decreased towards zero whereas the hydrostatic pressure increased The calculated net driving force was however, reduced to 8 ? mm Hg in spite of an increased amount of fluid reabsorbed during this condition indicating increased capillary hydraulic permeability

A model for the regulation of the renal interstitial hydrostatic and oncotic pressures will be presented and it is suggested that these parameters will influence the proximal tubular transport

Reference

Wiederhielm C A J W Woodbury S Kirk and R F Rushmer *Am J Physiol* 1964 207 173—176

COMMUNICATIONS

C 1

Beta adrenergic Regulation of N acetyltransferase Activity in Rat Pineal Gland Organ Culture

By M BACKSTROM and L WETTERBERG *Psychiatric Research Center Ulleraker Hospital University of Uppsala Sweden*

This study illustrates how a drug mimicks the effect of a neurotransmitter on the regulation of postsynaptic metabolism

Serotonin (5 hydroxytryptamine) is enzymatically metabolized by N acetyltransferase to N acetylserotonin It is converted to the pineal gland hormone melatonin by O methylation The N acetyltransferase activity may be induced by stimulation of a β adrenergic receptor mechanism (Klein and Weller 1972) In the present experiment we tested the ability of the β stimulating drug terbutaline (INN) (1 (3 5 dihydroxyphenyl 2(t butylamino) ethanol) to induce N acetyltransferase activity and increase melatonin formation *in vitro*

The experimental conditions used in this study have been described elsewhere (Backstrom and Wetterberg 1972) Condensed Sprague Dawley male rats were kept on a diurnal lighting schedule The animals were sacrificed 4 h after the onset of light Pineal glands were placed on nets in watch glasses containing culture medium at 37° C in 95 % O Terbutaline was added to the media and the glands incubated for 6 h The N acetyltransferase enzyme activity was assayed according to Klein Berg and Weller (1970) Addition of terbutaline or noradrenaline caused an approximately 10 fold increase in the N acetyltransferase activity in the rat pineal gland (Table I Terbutaline induction of N acetyltransferase occurs also in denervated pineal glands devoid of noradrenaline (Table I)

Some pineal glands were incubated for 24 h with ^{14}C serotonin and terbutaline added to the media The media were analysed for isotope labelled serotonin me

TABLE I The effect of noradrenaline and terbutaline on N acetyltransferase activity in the pineal gland organ culture for 6 h

Treatment	Conc in media (M)	N	N acetyltransferase activity in picomoles product formed per gland per hour
Control		(12)	148 ± 29
Noradrenaline	10^{-}	(4)	11 0 ± 160*
Terbutaline	10^{-}	(4)	1401 ± 121
SCG ect + Terbutaline	10^{-}	(4)	2947 ± 180**

The results are expressed as mean ± S E
SCG ect superior cervical ganglionectomy was carried out 3 weeks prior to the experiment
* Significantly different from control values $p < 0\ 001$ (Student s t test)
** Significantly different from the group of innervated pineals stimulated with terbutaline 10^{-} M ($p < 0\ 001$)

tabolites (Klein and Notides 1969) Terbutaline causes a higher concentration of N acetylserotonin and melatonin as compared to control values The production of 5 HIAA (5 hydroxyindole acetic acid) was not affected by terbutaline

The results support the hypothesis that a β adrenergic receptor mechanism takes part in the regulation of serotonin metabolism in the rat pineal gland

References

BACKSTROM M and L WETTERBERG *Acta physiol scand* 1972 In press
KLEIN D C and A NOTIDES *Analyt Biochem* 1969 *31* 480—483
KLEIN D C G R BERG and J WELLER *Science* 1970 *168* 979—980
KLEIN D C and J WELLER 1972 *J Pharm Expt Ther* In press

C 2

Rapid Effect of Ventromedial Hypothalamic Lesions on Lipogenesis in Rats

By B E HUSTVEDT and A LOVO *Nutrition Institute Physiology Branch University of Oslo Blindern Norway*

Hyperphagia and extensive accumulation of body fat are the most conspicious effects of ventromedial hypothalamic (VMH) lesions in the adult rat This is also reflected in the changed metabolism of the obese hypothalamic hyperphagic animal promoting lipid deposition with hyperinsulinaemia as one of its most pronounced features (Hales and Kennedy 1964) These metabolic changes are all known to develop in the normal organism as an adaptation to increased food intake and obesity In searching for metabolic changes due to the VMH lesion *per se* it has therefore been difficult to separate secondary adaptations from the primary effects During the last years evidence has accumulated indicating metabolic disorders following VMH lesions without concomitant hyperphagia Weanling rats which do not become hyperphagic after VMH lesions have been shown to deposite more fat than the controls when tubefed equal amounts of food (Frohman *et al* 1969) This is true also in the hypophysectomized VMH lesioned animal compared to hypophysectomized controls These findings exclude the hypophysis as the mediator of these effects (Han and Frohman 1970) It is important therefore to study metabolic changes which may appear during the first hours following VMH destruction in order to get further information as to the nature and ethiology of the obesity caused by the VMH lesion

We have studied the *in vivo* lipogenesis in rats during the first 8—12 h after VMH lesion The incorporation *in vivo* of ^{3}H labelled water into lipids from parametrial fat pads was measured in untreated and VMH lesioned animals During the *in vivo* incubation the animals had access neither to food nor water The results are given as relative specific activity (RSA) defined as the specific activity of the lipids (cpm/mg) divided by the specific activity of the body water (cpm/μl)

Our results clearly demonstrate an increased lipid synthesis in lesioned animals compared to the controls expressed as a significant difference in RSA ($p < 0.001$) of parametrial lipids 12 h after VMH destruction One of the early metabolic adaptations to fasting is a decreased rate of lipid synthesis This is reflected among the

control rats in our experiments the RSA of their lipids obtained at 8 and 12 h being identical Contrary to the control animals however the RSA of the lipids from the lesioned animals suggests an increased rate of lipid synthesis during the same time period the increase being 3 fold

References

FROHMAN L A L L BERNARDIS J D SCHNATZ and L BUREK Amer J Physiol 1969 216 1496—1501
HALES C N and G C KENNEDY Biochem J 1964 90 620—624
HAN P W and L A FROHMAN Amer J Physiol 1970 219 1632—1636

C 3

Water Diuresis Induced by Infusions of Non electrolyte Solutions into the Cerebral Ventricular System of the Conscious Goat

By L ERIKSSON *Department of Physiology College of Veterinary Medicine Helsinki Finland*

The current concept that osmoreceptors in the hypothalamic region of the brain play an essential role in the regulation of the release of antidiuretic hormone (ADH) is based on Verney s (1947) investigations in the dog However the results of more recent studies of central control of fluid balance in the goat appear incompatible with the osmoreceptor theory Rather these studies (Andersson 1971 Eriksson Fer nandez and Olsson 1971 Olsson 1972) indicate that the release of ADH is influenced by the Na concentration of the cerebrospinal fluid (CSF) Therefore it was of in terest to study whether an artificially induced lowering of the CSF Na concentration would inhibit the release of ADH normally occurring in the non hydrated animal

Various isotonic or hypertonic (450—600 mOsm/kg) non-electrolyte solutions were slowly infused into the cerebral ventricular system of conscious goats for 40—60 min Infusions into the third ventricle were made at a rate of 10 or 20 μl/min and into the lateral ventricle at a rate of 20 or 50 μl/min. Solutions of the following substances were used d glucose d fructose sucrose glycerol and urea The infusions of all these solutions induced an increase in urine flow while urine osmolality de creased This increase in the renal clearance of free water (C_{H_2O}) began 20—30 min after start of the infusions into the third ventricle The response was somewhat more delayed during infusions into the lateral ventricle No obvious change occurred in the renal sodium excretion and o molar clearance during the infusions However when C_{H_2O} returned to pre infusion level an increase in renal Na excretion was sometime seen

The observed increase in renal C_{H_2O} in all probability was due to an inhibition of the normal release of ADH The fact that the response was obtained also to the in fusions of hypertonic solutions suggests that the ionic composition of the CSF is of greater importance for the release of ADH than are the strictly osmotic factors Thus the present experiments support the idea that a Na sensitive receptor system

near the third cerebral ventricle plays an important role in the control of fluid balance

References

ANDERSSON B *Amer Scientist* 1971 *59* 408—415
ERIKSSON L O FERNANDEZ and K OLSSON *Acta physiol scand* 1971 *83* 554—562
OLSSON K *Life Sci* 1972 *11* Part I 397—402
VERNEY E B *Proc roy Soc B* 1947 *135* 25—106

C 4

The Giant Axon of the Leech

By A R. GARDNER MEDWIN* J K S JANSEN and T TAXT *Institute of Physiology University of Oslo Norway*

Systems of giant axons have been described in several invertebrates They usually mediate teleologically meaningful escape behaviour We have studied a large unique axon in the leech central nervous system which defies any such simple interpretation of its function The axon is a single fibre which runs throughout the nerve cord in the medial unpaired connective (Bagnoli *et al* 1972) Its diameter is about 12 μm twice that of any other nerve fibre in the connectives One identifiable cell body in each of the abdominal ganglia is intimately connected to the giant axons Procion yellow injections show a direct continuity between the cell and the axon Action potentials are conducted along the axon throughout the nerve cord and invade the associated cell body in each ganglion

The segmental synaptic input to the system can be recorded intracellularly from the cell bodies Primary afferent neurones activated by light touch of the body surface (T cells Nicholls and Baylor 1968) are directly electrically coupled to the system and are also connected through a slower probably polysynaptic pathway Local segmental stimulation by light also excites the system and commonly fired the cell and axon within 100—200 ms

Efferent connections of the giant axon have been found to two different pairs of identified motor neurones controlling body wall musculature But the connections are weak and several giant axon impulses are required to fire the motoneurones The synapses between the giant axon and the two motoneurones are electrical In preparations with intact motor nerves even a high frequency burst of impulses elicited by an intracellular electrode produced only weak contractions of the longitudinal and anulus erector muscles (Stuart 1971) of the wall body

The action potential of the giant axon could be recorded reliably with implanted electrodes from an intact leech Bursts of impulses were elicited by stimulation with touch or light anywhere on the skin Impulses were conducted along the axon in either direction according to where the stimulus was applied The giant axon was quiet during swimming movements

* Royal Society European Exchange Fellow Present address Dept of Physiology University College London London WC1 E 6BT

In conclusion the single median giant axon of the leech forms a two way rapid longitudinal conduction system with local segmental inputs and outputs It is unlikely that it mediates escape responses as do the giant fibres of other annelids (Nicol 1948)

References

Bagnoli P M Brunelli and F Magni *Arch Ital Biol* 1972 *110* 35—51
Nicholls J G and D A Baylor *J Neurophysiol* 1968 *31* 740—756
Nicol J A C *Quart Rev Biol* 1948 23 291—323
Stuart A E *J Physiol* 1970 209 627—646

C 5

Locomotion in the Spinal Dogfish

By S Grillner *Department of Physiology University of Goteborg and Kristineberg Zoological Station Fiskebackskil Sweden*

After spinalization some elasmobranches particularly the dogfish are unique in that the body exhibits coordinated spontaneous locomotion (Lissman 1946) During swimming the two sides of each segment contracts alternately and the frequency is changed with the speed of swimming As the fish swims an undulating wave passes along the body

Nine *Squalus acanthiasis* were spinalized 5 to 9 segments below the cranium During recording the head was fixed while the body was free to move EMG activity was recorded on an 8 channel Mingograph During slow swimming EMG activity could be recorded in the red lateral muscle of the body whereas no activity was revealed in the white muscles During faster swimming rhythmic activity in phase with that of the red muscles could easily be recorded both in the dorsal and ventral white muscles Thus these muscles do participate in swimming which has been questioned by Bone (1966) and Roberts (1969) To see the relation between the frequency of alternation in each segment and the speed with which the undulatory wave travels along the body 6 electrodes were placed in different segments along the body During forward swimming cranial segments were activated prior to more caudal ones

The rate with which the undulatory wave travelled *from segment to segment* was constant at any frequency (of tail beat) but varied on the other hand greatly with this same frequency The time to travel 10 segments could be 25 ms with a duration of the EMG discharge in one segment of 200 ms but 125 ms with a discharge of 800 ms There is a linear relation between these two parameters from the lowest rates of swimming to the highest Thus there is a phase lag between each segment which is independent of the speed of swimming i e the wave form of the body will remain the same independently of the tail beat frequency The results agree with cinematographical studies (Kashin personal communication)

I *interpret the results of* Lissman (1946) Roberts (1969) von Holst (1935) and others as suggesting that the rhythm generation is in the CNS itself and not dependent on chain reflexes Further sections of the spinal cord were performed in parts

containing down to 8 segments Any of these could independently generate alternating activation of the two sides and sequential activation of consecutive segments. Hence locomotor activity can be generated in any part of the spinal cord The most plausible explanation of the present findings is that each segment has a central rhythm generator an oscillator The oscillator of one segment is phase coupled with that of the subsequent and o forth

References

Bone Q *J mar biol Ass U K* 1966 *46* 321—349
Holst E von *Pflugers Arch ges Physiol* 1935 *236* 149—158
Lissman H W *J exp Biol* 1946 *23* 162—176
Roberts B L *J mar biol Ass U K* 1969 *49* 33—49

C 6

Hydrodynamic Properties of Kidney Tubules during Ethacrynic Acid Administration

By F Kiil P Ostvik Jr and M Reder *Institute for Experimental Medical Research University of Oslo Ulleval Hospital Norway*

Most of the hydrodynamic resistance of the nephron is probably localized to the distal nephron as no pressure gradient has been demonstrated along the proximal tubules (Gottschalk and Mylle 1956) Renal tubules dilate during osmotic diuresis (Gottschalk and Mylle 1957) and tubular hydrodynamic conductance accordingly increases When ethacrynic acid is infused i v renal tubular dimensions expand as the diuretic effect is established and remain expanded and largely constant when urine flow is subsequently reduced by lowering of renal perfusion pressure (Ostvik *et al* 1971) Since ethacrynic acid severely curtails reabsorption in the distal segments o the nephron—and thereby probably expands the tubules—it is possible that the istal tubules might exhibit high and constant hydrodynamic conductance under ethacrynic acid diuresis The purpose of the present study was to determine whether the overall tubular hydrodynamic conductance might approach a maximum during ethacrynic acid infusion

In 12 anesthetized dogs undergoing ethacrynic acid diuresis and with control glomerular filtration rate averaging 56 7 ± 4 2 ml/min the relationship was examined between urine flow (V) and the difference (P) between intrarenal tissue pressure (IRP) in the cortex and ureteral pressure Tentatively $\Delta V/\Delta P$ was used as an estimate of tubular hydrodynamic conductance

Urine flow was varied by 3 procedures aortic constriction above the renal artery tepwise ureteral constriction and intravenous infusion of mannitol or large quantities of saline Under each procedure almost similar $\Delta V/\Delta P$ was obtained averaging 0 76 ml/min mm Hg during aortic constriction and 0 75 ml/min mm Hg after infusing saline or mannitol The corresponding ratio during ureteral constriction averaged 0 72 ml/min mm Hg There was no change in urine flow or IRP before ureteral pressure exceeded 10—15 mm Hg The constant $\Delta V/\Delta P$ could not be at

tributed specifically to changes in arterial perfusion pressure filtration rate or renal blood flow

It is concluded that after ethacrynic acid administration the nephrons behave like expanded tubes of high and constant hydrodynamic conductance

References

GOTTSCHALK C W and M MYLLE Am J Physiol 1956 185 430—439
GOTTSCHALK C W and M MYLLE Am J Physiol 1957 189 323—328
OSVK P JR. M RÆDER and F KIIL Am J Physiol 1971 221 1560—1567

C 7

Distribution of Red Cells and a Macroprotein in the Rat Kidney during Antidiuresis and Osmotic Diuresis

By S NYBO RASMUSSEN *Institute of Medical Physiology B University of Copenhagen Denmark*

Intrarenal red cell and plasma volumes may be estimated by means of labelled red cells and a suitable plasma tracer The total amounts of tracers in tissue and the arterial concentrations per volume red cell and plasma respectively are determined after a certain period of equilibration following i v injection It is however difficult to find a plasma tracer to which the renal blood vessels are impermeable (Ulfendahl 1962 *Pinter 1967 and Kompf et al 1971*) In the present study ^{51}Cr labelled rat red cells and ^{125}I human γ M globulin (M = 920 000) were used In two series of experiments 1 and 10 min respectively elapsed from i v injection of the tracers until clamping of the pedicle and freezing of the kidney Except for a slight increase with time in the cortical plasma volume the distribution volumes did not differ between the two series During antidiuresis the red cell and plasma volumes of the whole kidney were 6 0 ± 0 9 (mean ± S D) ml and 9 1 ± 1 3 ml per 100 g kidney respectively Outer and inner medulla had significantly higher red cell and plasma volumes than cortex

The intrarenal hematocrits calculated from the distribution volumes were compared with the arterial hematocrit (aorta) and the total body hematocrit (Table I) The intrarenal hematocrit in all zones was significantly lower than the total body hematocrit and the aortic hematocrit In the inner medulla the hematocrit was considerably lower than in the other renal zones The hematocrit in all zones was lower during mannitol diuresis than during antidiuresis *Provided that no significant*

TABLE I Intrarenal and aortic hematocrits expressed *relative* to the total body hematocrit* (mean values ± S D)

	whole kidney	cortex	outer medulla	inner medulla	aortic blood
Antidiuresis n = 11	0 93 ± 0 08	0 89 ± 0 09	0 93 ± 0 11	0 62 ± 0 17	1 16 ± 0 06
Mannitol diuresis n = 10	0 81 ± 0 13	0 78 ± 0 13	0 80 ± 0 15	0 41 ± 0 14	1 17 ± 0 06

All figures represent experiments with 1 min tracer equilibration

amounts of γ M globulin escaped from the renal vessels within the first minute after injection the results indicate that this macroprotein is suitable as an intravascular tracer in the kidney

References

KOMPF B K P KARSUNKY and R. TAUGNER *Pflugers Arch ges Physiol* 1971 *328* 205–220
PINTER G G *J Physiol* (Lond) 1967 *192* 761—772
ULFENDAHL H R *Acta physiol scand* 1962 *56* 42—60

C 8

Intrarenal Distribution of Glomerular Filtration in Conscious Rats during Isotonic Saline Infusion and Hemorrhagic Hypotension

By G CLAUSEN and I TYSSEBOTN *From the Institute of Physiology University of Bergen Norway*

The purpose of this investigation was to study the possible heterogeneity of filtration rate in superficial and juxtamedullary nephron populations during i v saline loading and hemorrhagic hypotension

The overall rate of tubular Na reabsorption is depressed during volume expansion by i v saline infusion It has been suggested that a redistribution of filtrate from deep to superficial nephrons with relatively low Na reabsorption capacity might disrupt the glomerotubular balance of these nephrons and thus cause the natriuresis characteristic of volume expansion On the other hand total GFR is greatly reduced during volume depletion and hemorrhagic hypotension There is however a persistent disagreement as to whether this results in altered distribution of filtrate within or between the two nephron populations We therefore determined the relative individual glomerular filtration rate (gfr) in the two types of nephrons during control condition i v saline loading and hemorrhagic hypotension using the single bolus ^{14}C ferrocyanide technique

Isotonic saline infused at 250 μl/min 100 g b wt for about 60 min produced an average effective volume expansion of 5 5 % Fractional Na reabsorption was reduced by 11 % with no significant change in inulin clearance The superficial gfr was 90 % of juxtamedullary gfr both in control and saline loaded animals but with less scatter in the latter group Consequently the natriuresis produced by acute i v isotonic saline loading in conscious rats was not caused by altered intrarenal distribution of filtrate

Hemorrhagic hypotension was produced by bleeding from the femoral artery A mean arterial pressure ($\overline{AP}$) of 50 and 70 mm Hg was maintained for 120 min in 2 groups of rats Hypotension drastically reduced the average amount of ferrocyanide in individual tubules as compared to controls No filtration could be detected in a major fraction of the nephrons at $\overline{AP}$ 50 mm Hg At $\overline{AP}$ 70 mm Hg individual tubular ferrocyanide ranged from zero to near control levels and non filtering nephrons were reduced to about 10 % of total At both pressure levels the fraction

of non filtering nephrons as well as the distribution of ferrocyanide were similar in superficial and juxtamedullary nephrons Thus neither i v isotonic saline loading nor hemorrhagic hypotension altered the distribution of filtrate between deep and superficial layers of the renal cortex in conscious rats

C 9

3 O Methylglucose Flux in Isolated Fat Cells Effect of Insulin and Phlorizin

By J Gliemann K. Østerlind and J Vinten *The Institute of Medical Physiology C University of Copenhagen Denmark*

The effects of insulin phlorizin, temperature and 3 0 methylglucose concentration on the equilibrium efflux of ^{14}C labelled 3 0 methylglucose from isolated rat fat cells were studied using a recently described technique (Gliemann *et al* 1972) The cells were equilibrated for 1 h at 37° C with 3 0 methylglucose 0 5 mM and 3-0 ^{14}C methylglucose in a trace concentration At this time the intracellular 3 0 ^{14}C methylglucose space was equal to the intracellular ^3H_2O space The medium was then removed and the packed cells were diluted at 0 time in buffer containing un labelled 3 0 methylglucose 0 5 mM but no 3 0 ^{14}C methylglucose

Fig 1 shows that the efflux of 3 0 ^{14}C methylglucose had to be described as the sum of at least 2 exponential functions The half time for the efflux from the slowly emptied compartment was about 100 s and remained unchanged in the above men tioned test situations The half time of the rapidly emptied compartment could not be determined since the first experimental point was taken 30 s after removal of the extracellular 3 0 ^{14}C methylglucose With insulin 10^4 μU/ml the fat cells contained less 3 0 ^{14}C methylglucose after 30 s than the control cells and with phlorizin 2 mM

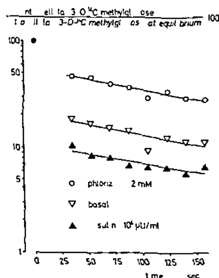


Fig 1 *Effect of insulin and phlorizin on equilibrium efflux of 3-0 ^{14}C methylglucose in isolated fat cells* The intracellular amount of 3 0 ^{14}C methylglucose at equilibrium ● was determined with 8 replicates

they contained more Increase of the 3 0 methylglucose concentration to 80 mM or decrease of the temperature to 20° C had effects similar to that of phlorizin 2 mM

The observed changes might theoretically be caused by changes in compartment size and/or in the rate constant for efflux from the rapidly emptied compartment *If it is assumed that only the rate constant changes the results may be interpreted as* follows Insulin increases and phlorizin decreases carrier facilitated flux across the cell membrane which constitutes the barrier of the rapidly emptied compartment Another compartment present within the cell, is emptied at a relatively slow rate and possibly by the process of free diffusion

Reference

GLIEMANN J K ØSTERLIND J VINTEN and S GAMMELTOFT *Biochim biophys Acta* 1972 *In print*

C 10

Lipid Digestion in the Stomach—a Process of Physiological Importance in the Suckling Rat

By A BILLSTROM and T OLIVECRONA *Departments of Anatomy and Physiological Chemistry University of Umea Sweden*

In the suckling rat the lipid content of milk comprises about 80 % of the calories available Helander and Olivecrona (1970) have previously demonstrated an extensive lipolysis of milk lipids in the stomach of the suckling rat the products being partial glycerides predominantly diglycerides and fatty acids It was also shown (Egelrud Olivecrona and Helander 1971) that these liberated fatty acids can be rapidly absorbed in the stomach and transferred to the vascular system for subsequent utilization in the tissues Recently Hamosh and Scow (1972) described an acid stable lipase (pH optimum 4) in the von Ebners gland of the rat tongue which may be *responsible for the lipolysis in the stomach of the suckling rat* where the content has a pH between 4 and 6

von Ebners glands were homogenized in 0 01 M Tris HCl pH 7 4 followed by centrifugation at 5 5 10^6 g min Almost all enzyme activity sedimented *When the* pellet was resuspended in the same buffer but at pH 8 5 the enzyme was *obtained in solution Thus extract showed lipolytic activity between pH 3 and 8 against triolein* Intralipid® tributyrin and the water soluble substrate p nitrophenylacetate

The significance of milk lipolysis in the stomach can be looked upon in at least two different ways Firstly part of the ingested lipids are made available for early absorption Secondly the gastric lipolysis may transform the milk droplets to a form more suitable for the subsequent lipolysis *in the small intestine* Preliminary evidence in favour of this suggestion has been obtained by *in vitro* studies Rat milk was incubated at pH 6 in a weak potassium phosphate buffer, containing 4 mM taurocholic acid Enzyme extract from von Ebners gland or a pancreatic homogenate was added resulting in considerable lipolysis of the milk When the milk was incubated first with

the von Ebner s gland extract for five minutes followed by the addition of pan creatic homogenate an acceleration of the lipolytic activity occurred the total rate exceeding about twice the sum of the activity obtained by incubating the two enzyme preparations alone

References

EGELRUD T T OLIVECRONA and H F HELANDER *Scand J Gastroenterology* 1971 *6* 329—333
HAMOSH M and R O SCOW *J Clin Invest* 1972 In press.
HELANDER H F and T OLIVECRONA *Gastroenterology* 1970 *59* 22—35

C 11

Intensity Function of Cold Receptors with Slow Temperature Changes

By H HENSEL and T JÄRVILEHTO *Institute of Physiology University of Marburg West Germany*

In cat the intensity function of cold receptors produced by very fast cold stimuli is linear up to 8° C and then curved (Hensel 1953) Slow temperature changes (0 4° C/s) on the other hand are associated with a curvilinear function (Kenshalo *et al* 1971) In the present work the intensity function of a cold receptor was re-examined by applying linear cold stimuli which were four times faster than those of Kenshalo *et al* (1971) and covered a larger range of stimulus intensities Such stimuli should produce a function more linear than that of Kenshalo *et al* (1971) if the form of the intensity function is consistently dependent on the speed of the temperature change

The branch of the infraorbital nerve of the cat supplying the nose was used for single fiber preparations The stimuli were delivered by a thermode of 1 mm dia meter In a stimulus series the temperature of the thermode was changd from 30° C with a rate of 1 6° C/s to 6 different levels (1 stimulus/2 min) and returned back to the adapting temperature Stimulus duration was maximally 6 s Stimulus tempera ture was recorded with a copper constantan thermocouple at the tip of the thermode The average number of spikes per second during the dynamic period (a period from the acceleration of the firing frequency to the beginning of the adaptation) was calculated

45 receptors were quantified (26 in the hairy and 19 in the glabrous skin) The frequency of the cold fibers was curvilinearly dependent on the stimulus intensity the function reaching a plateau with stimuli larger than 6° C The function was less curved than that described by Kenshalo *et al* (1971) A difference between the receptors located in the hairy and in the glabrous skin was found the former having steeper intensity functions and higher response frequencies

The present results are consistent with earlier findings The intensity function of cold receptors seems thus to turn more linear with increasing rate of temperature change but reaches in any case a plateau with cold stimuli exceeding 6—8° C

References

HENSEL H *Pflugers Arch ges Physiol* 1953 256 470—487
KENSHALO D R H HENSEL P GRAZIADEI and H FRUHSTORFER In Oral facial sensory and motor mechanisms Eds R DUBNER and Y KAWAMURA New York Appleton Century Crofts 1971 pp 23—45

C 12

Ionic Effects on Adaptive Behaviour of Crustacean Stretch Receptor Neurons

By D OTTOSON *Department of Physiology Royal Veterinary College, Stockholm Sweden*

Recent studies have provided evidence that the early adaptation of the frog muscle spindle is related to the ionic events underlying the transducer action of the sensory endings (*cf* Ottoson 1972) These observations suggested that the adaptation of other types of slowly adapting mechanoreceptors might be of similar nature This hypothesis has been tested by exposing isolated crustacean stretch receptor neurons (*Astacus fluviatilis*) to potassium free solutions The receptor muscle was attached at each end to a fine nylon rod Stretches were applied with an electromagnet (Husmark and Ottoson 1971) to both ends of the muscle The receptor potential was recorded extracellularly (*cf* Eyzaguirre and Kuffler 1955) after blocking the impulse activity with tetrodotoxin

Removal of potassium from the external solution produced a sequence of changes in time course and amplitude of the different phases of the response to a given stretch (Fig 1) In the rapidly adapting cell the dynamic overshoot decreased and was usually abolished after 60 to 90 min The static phase suffered an initial reduction which was followed by an increase and at the time when the dynamic overshoot had disappeared the static phase had usually regained its original amplitude or become somewhat higher The falling phase of the response following release of stretch was greatly prolonged The dynamic phase of the slowly adapting neuron underwent similar changes with the exception that the overshoot was not completely

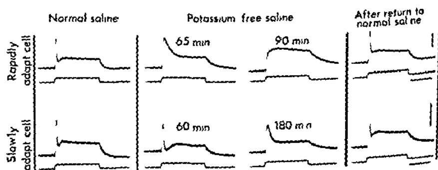


Fig 1 Effect of removal of potassium on receptor potential of crustacean stretch receptor neurons Extracellular recordings of receptor responses to step stretches (lower trace) after blocking of conducted activity with tetrodotoxin (2×10^{-7} g/ml) Time calibration 50 ms Vertical bars upper row 1 mV lower row 0 5 mV

abolished even with prolonged (3—4 h) exposures of the neuron to the potassium free solution The static phase was generally reduced in amplitude

These observations suggest that the adaptive fall of the receptor potential of the rapidly adapting cell is mainly determined by the ionic processes in the transducer membrane while in the slowly adapting neuron other mechanisms also contribute to the adaptive fall of the response

References

Eyzaguirre C and S W Kuffler *J Gen Physiol* 1955 *39* 87—119
Husmark I and D Ottoson *Acta physiol scand* 1971 *83* 486—494
Ottoson D *In* Research in muscle development and the muscle spindle *Ed* B Q Banker *et al* *Excerpta Medica* 1972 341—352

C 13

Dynamic Properties of Stretch induced Chronotropy in the Isolated Heart of the Snail Helix pomatia

By M Almqvist *Institute of Physiology and Medical Biophysics Biomedical Center University of Uppsala Sweden*

Intracardiac diastolic pressure variations and external mechanical stimulation have chronotropic effects on isolated hearts (Jensen 1971) Properties of this stretch related chronotropy under static conditions are quite well known Very few investigations have dealt however with the response to cyclical tension variations Some authors have noticed that the velocity of stretch influences the degree of pace maker acceleration (Brooks and Lu 1972)

In the present investigation the response to stretch was recorded under dynamic conditions Isolated spontaneously beating ventricle strips from snail hearts were used Sinusoidal length variations were produced by a moving coil puller One end of the strip was mounted to the puller and the other end to a force transducer The position of the movable end was measured by a displacement transducer Suction electrodes were used for monophasic action potentials Heart rate was measured by an instantaneous ratemeter

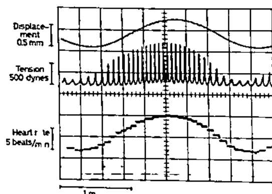


Fig 1 Positive inotropic and chronotropic effects of sinusoidal displacement

A representative recording is shown in Fig 1 A small sinusoidal displacement causes a marked acceleration and an augmentation of contractile force The sensitivity to stretch is frequency dependent, being low at low frequency input to the puller The phase difference between length and heart rate is also influenced by the frequency

The sensitivity is modified by the displacement amplitude and by the initial diastolic tension To some extent, the non linearity of the input-output relation is related to the visco-elastic properties of the tissue The pacemaker potential re cordings show that the slope of the slow diastolic depolarization increases in steepness during stretching

References

Brooks C Mc C and H H Lu The sinoatrial pacemaker of the heart *Charles C Thomas Publisher* Springfield Ill 1972

Jensen D Intrinsic cardiac rate regulation. *Appleton Century Crofts* New York N Y 1971

C 14

Reflex Suppression of Gastric Motility Elicited from Antral Mechanoreceptors

By H Abrahamsson *Department of Physiology University of Goteborg Swe den*

Gastric mechanoreceptors are involved in the control of hunger and satiety, and gastric secretion (see *e g* Sharma 1967) but their role in regulation of gastric motility is unclear In the present study it was explored whether activation of mechanoreceptors in the gastric antrum can reflexly affect gastric motility by ac tivation of the two different extrinsic suppressive nerve supplies to the stomach the vagal non adrenergic relaxatory fibres and the splanchnic adrenergic fibres (Jan n 1969)

In anesthetized cats the corpus fundus part of the stomach was separated from the antrum by a ligature and the motility in the corpus fundus part was recorded by a volume method Slight antral distension by a pressure of 8—20 cm H O had 2 different but concomitant effects on the corpus fundus motility Firstly a pro nounced long lasting relaxation was recorded with marked increase of the corpus-fundus volume This response persisted after administration of guanethidine and atropine but was abolished by vagotomy Secondly antral distention produced an inhibition of rhythmic contractile corpus fundus waves induced by electric stimu lation of the distal ends of the cut vagal nerves This type of inhibition was also observed when the vagi were blocked by cooling and was abolished by guanethidine and by lidocaine blockade of the splanchnic nerves

It is concluded that slight distension of the gastric antrum suppresses gastric motility by two different mechanisms a vago vagal reflex with the vagal relaxatory fibres as the efferent link and a reflex *via* splanchnic adrenergic fibres which in hibits excitatory gastric motility Both reflex mechanisms have considerable effects and acting together they can effectively suppress gastric motility The described re

flex mechanisms are suggested to be involved in the physiological control of gastric reservoir and emptying functions

References

JANSSON G *Acta physiol scand* 1969 Suppl 3?6 1—42
SHARMA K N *Handbook of physiology* Sect. 6 Vol II 1967 pp 225—?37

C 15

Analogous Effects of Cholecystokinin and Prostaglandin E on Mechanical Activity and Tissue Levels of cAMP in Biliary Smooth Muscle

By K E ANDERSSON R ANDERSSON P HEDNER and C G A PERSSON
Department of Medicine University Hospital Lund Department of Pharmacology Linkopings Högskola Linkoping and Draco AB Lund Sweden

In the gall bladder the contraction induced by the C terminal octapeptide of cholecystokinin (C8 CCK) was found to be preceded by an activation of phosphodiesterase (PDE) and a reduction of the content of cyclic adenosine 3 5 monophosphate (cAMP) in the tissue In the sphincter of Oddi the relaxation induced by C8-CCK was preceded by an increase in the cAMP content, probably caused by an activation of adenyl cyclase (Andersson *et al* 1972)

The ubiquitous prostaglandins which also affect gastrointestinal motility have been suggested to be local modulators of hormonal effects by interfering with the tissue cAMP levels (Shio *et al* 1971) To test the possibility that prostaglandins may be involved in the actions of C8 CCK we found it of interest to compare the effects of some prostaglandins on biliary smooth muscle with those of C8 CCK

Gall bladder strips from guinea pigs and longitudinal preparations of the sphincter of Oddi from cats were mounted in organ baths containing Krebs solution kept at 37° C and gassed with carbogen Isometric tension was recorded cAMP was determined according to Kakiuchi and Rall (1968) and PDE activity according to Kukovetz and Poch (1970)

Prostaglandins E_1 E and F were investigated Among these prostaglandin E (PGE) consistently contracted the gall bladder but relaxed the sphincter of Oddi as did C8 CCK These responses were unaffected by atropine and by alpha and beta receptor blocking agents Thirty seconds after the administration of PGE 4×10^{7} g/ml when the tension had just begun to increase in the gall bladder strip there was a 75 % decrease of the cAMP content and a 66 % increase of the PDE activity in the tissue After 180 s when the mechanical response was maximal these changes were 80 and 54 % respectively

When the relaxation of the sphincter of Oddi became visible 30 s after the addition of PGE 4×10^{7} g/ml there was a 50 % increase in the cAMP content and according to 4 preliminary experiments a 23 % increase in the PDE activity

These results show that in the gall bladder and in the sphincter of Oddi there is a close similarity in time course and magnitude of the mechanical and metabolic

responses to C8 CCK and PGE The question whether or not the effects of C8 CCK are mediated by a local synthesis and release of PGE_2 remains however, to be settled

References

ANDERSSON K E R ANDERSSON P HEDNER and C G A PERSSON *Acta physiol scand* 1972 *84* 19 A—20 A
KAKIUCHI S and T W RALL *Molecular Pharmacol* 1968 *4* 367—378
KUKOVETZ W R and G POCH *Arch Pharmacol exp Pathol* 1970 *267* 189—194
SHIO H J SHAW and P RAMWELL *Ann N Y Acad Sci* 1971 *180* 327—335

C 16

Dissociation of Prostaglandin and α Receptor Mediated Control of Adrenergic Transmitter Release

By P HEDQVIST *From the Department of Physiology Karolinska Institutet Stockholm Sweden*

During recent years 2 mechanisms for the control or noradrenaline (NA) release have been advanced one prostaglandin (PG) mediated (Hedqvist 1970) and the other based on activation of possibly prejunctionally located α receptors (Haggendal 1970) Both hypotheses have received considerable support It was therefore decided to test if the proposed mechanisms are linked together or represent 2 independent means for modulation of transmitter release

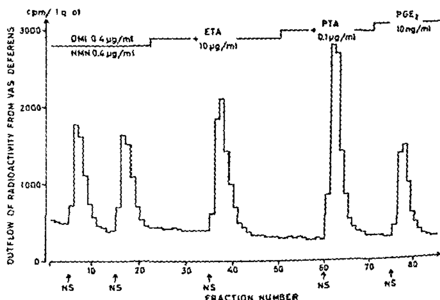


Fig 1 Isolated superfused guinea pig vas deferens loaded with ^3H NA Outflow of radioactivity from the preparation resting and in response to transmural stimulation (NS) (450 pulses 5 Hz 1 ms) Desmethylimipramine (DMI) and normetanephrine (NMN) present throughout the experiment Effect of additional administration of eicosatetraynoic acid (ETA) phentolamine (PTA) and PGE_2

Guinea pig vasa deferentia, preincubated with ^3H NA were superfused with Tyrode in an organ bath and subjected to transmural stimulation (450 pulses 5 Hz 1 ms supramaximal voltage) NA recapture was inhibited with desmethylimipramine (DMI) and normetanephrine (NMN) The superfusate was fractionated (1 ml/min) and analyzed for radioactivity Administration to the bath of 5 8 11 14 eicosa tetraynoic acid (ETA) 10 μg/ml consistently and significantly increased the outflow of radioactivity (NA) from the stimulated vas deferens (Fig 1) and meanwhile blocked the efflux of PG like material suggesting that ETA enhances NA release by inhibiting PG synthesis The promoting action of ETA on NA release was completely abolished by PGE 10 ng/ml

Administration of phentolamine (0 1 μg/ml) in the presence of DMI NMN and ETA further considerably increased the outflow of radioactivity in response to nerve stimulation This effect of phentolamine was completely abolished by PGE_2 10 ng/ml (Fig 1) Closely similar results were obtained when phentolamine was replaced by Hydergine Since both reuptake of released NA and generation of PG were inhibited by prior administration of DMI, NMN and ETA it is suggested that α receptor blockade *per se* can remove a discrete not PG mediated mechanism operated to modulate transmitter release to forthcoming nerve action potentials

References

HAGGENDAL J *Bayer-Symposium II* pp 100—109 Springer Verlag 1970
HEDQVIST P *Acta physiol scand* 1970 Suppl 345 1—40

C 17

Relation between Splanchnic Ethanol Elimination and Oxygen Consumption

By N KRARUP and J A LARSEN *Institut of Physiology University of Aarhus Denmark*

It is generally accepted that ethanol is eliminated at a constant rate when present in the blood in concentrations higher than 1—2 mM Attempts to increase the elimination rate have so far only been successful with fructose administration Preliminary experiments from our laboratory demonstrated however that also glycine and alanine are able to increase the elimination rate

In the present experiments the effect of glycin and alanine on splanchnic elimination of ethanol and consumption of oxygen was investigated in 12 fasting cats Furthermore the redox state of the liver cell cytoplasm and mitochondria was estimated from the hepatic venous lactate/pyruvate and β hydroxybuturate/acetoacetate ratios respectively After a control period of 150 min an infusion of glycine or alanine corresponding to 0 1 gN/kg b wt was given in the course of 10 min

It appears from Fig 1 that both amino acids increase the splanchnic elimination of ethanol and consumption of oxygen In the control period as well as after amino acid infusion the splanchnic elimination rate of ethanol and consumption of oxygen

responses to C8 CCK and PGE The question whether or not the effects of C8 CCK are mediated by a local synthesis and release of PGE remains, however to be settled

References

ANDERSSON K E R. ANDERSSON P HEDNER and C G A PERSSON *Acta physiol scand* 1972 *84* 19 A—20 A
KAKIUCHI S and T W RALL *Molecular Pharmacol* 1968 *4* 367—378
KUKOVETZ W R. and G POCH *Arch Pharmacol exp Pathol* 1970 *267* 189—194
SHIO H J SHAW and P RAMWELL *Ann N Y Acad Sci* 1971 *185* 327—335

C 16

Dissociation of Prostaglandin and α Receptor Mediated Control of Adrenergic Transmitter Release

By P HEDQVIST *From the Department of Physiology Karolinska Institutet Stockholm Sweden*

During recent years 2 mechanisms for the control or noradrenaline (NA) release have been advanced one prostaglandin (PG) mediated (Hedqvist 1970) and the other based on activation of possibly prejunctionally located α receptors (Haggendal 1970) Both hypotheses have received considerable support It was therefore decided to test if the proposed mechanisms are linked together or represent 2 independent means for modulation of transmitter release

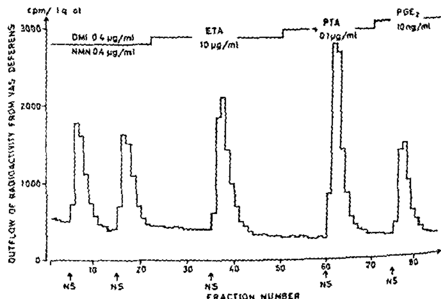


Fig 3 Isolated superfused guinea pig vas deferens loaded with ^3H NA Outflow of radioactivity from the preparation resting and in response to transmural stimulation (NS) (450 pulses 5 Hz 1 ms) Desmethylimipramine (DMI) and normetanephrine (NMN) present throughout the experiment Effect of additional administration of eicosatetraynoic acid (ETA) phentolamine (PTA) and PGE_2

during infusion of nicotine may be attributed to a concomitant increase in FFA Inhibition of lipolysis with β pyridyl carbinol (Ronicol® Hoffmann La Roche) abolishes the nicotine induced increase in FFA In the present study MVO was compared at intact and inhibited lipolysis during nicotine infusion

Seven intact mongrel dogs were anesthetized with pentobarbital and myocardial blood flow was measured by the hydrogen desaturation technique (Aukland *et al* 1964) Cardiac output (CO) was determined by thermodilution using a thermistor introduced into the aorta A transducer connected to a radiopaque catheter inserted into the left ventricle from a carotid artery was used to measure left ventricular pressure (LVP) and its first derivative (dP/dt) Another transducer connected to a catheter introduced into the aorta from a femoral artery recorded mean aortic blood pressure ($\overline{AP}$) Arterial and coronary sinus blood was sampled simultaneously and analysed for oxygen saturation, hemoglobin and FFA

Following control measurements stable hemodynamic conditions were reached after 5 min infusion of nicotine at an average dose of 15 μg/kg b wt min Lipolysis was then inhibited by continuous infusion of Ronicol and measurements were repeated before and during a new infusion of nicotine

Nicotine administration increased MVO by $4\,1 \pm 0\,9$ ml/100 g min before and $2\,1 \pm 0\,6$ ml/100 g min after inhibited lipolysis ($p < 0\,005$) With intact lipolysis arterial concentrations of FFA increased by 34 ± 4 % and myocardial uptake of FFA by 86 ± 23 % *No increase was observed in FFA during nicotine infusion at inhibited* lipolysis The increased myocardial performance as evidenced by increased LVP dP/dt CO and $\overline{AP}$ was essentially similar however during nicotine infusion before and after inhibited lipolysis

It is concluded that with intact lipolysis approximately 50 % of the increase in MVO during infusion of nicotine is attributable to increased mechanical activity while the remaining 50 % is due to increased oxygen demand induced by the increased myocardial uptake of FFA

References

AUKLAND K B BOWER and R W BERLINER *Circulat Res* 1964 *14* 164—187
KJEKSHUS J A S BELLET E R DICKSTEIN and L J FEINBERG *Circulat Res* 1961 *9* 631—638
MJOS O D *J Clin Invest* 1971 *50* 1386—1389

C 19

Control of Facilitation at the Neuromuscular Junction of the Lobster

By E FRANK *Department of Neurobiology Harvard Medical School U S A and Institute of Physiology University of Oslo Norway*

Synapses in addition to mediating the well studied rapid electrical signalling between excitable cells are also the sites of longer lasting interactions In the present study the experimental findings suggest that an individual muscle fiber may in

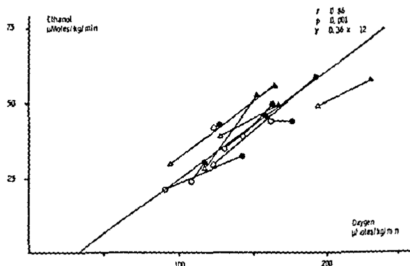


Fig 1 The relationship between splanchnic oxygen consumption and ethanol elimination before (open symbols) and after (closed symbols) glycine (circles) and alanine (triangles) Results from same experiment are connected by lines The regression line (y = 0 36 x − 12) is drawn

was significantly correlated the relation being the same before and after amino acids The intercept of the regression line on the abscissa (35 µmol/kg/min) is interpreted as the oxygen consumption in non ethanol metabolizing splanchnic organs ie intestines and spleen This was confirmed by 4 measurements of the pre hepatic splanchnic oxygen consumption yielding a mean value of 40 µmol/kg/min The slope of the regression line indicates that 36 % of the oxygen consumed by the liver is used for ethanol oxidation

In accordance with the increase in oxygen consumption the amino acids changed the mitochondrial redox state towards a more oxidized level whereas no significant effect on the cytoplasmic redox state was observed The discrepancy between the effect on cytoplasmic and mitochondrial redox levels may be explained by the increase in ethanol elimination which increases the production of reducing equivalents in the hepatic cytoplasm and thereby maintains the cytoplasmic redox state at the control level

It is concluded that the elimination rate of ethanol is dependent on the overall hepatic metabolism and may be regulated by the mitochondrial oxidation rate

C 18

Nicotine and Myocardial Oxygen Consumption

By A Ilebekk and O D Mjos *Institute for Experimental Medical Research University of Oslo Ulleval Hospital Oslo Norway*

Nicotine is known to increase plasma free fatty acids (FFA) (Kershbaum et al 1961) and elevation of FFA has been shown to increase myocardial oxygen consumption (MVO) without influencing mechanical activity of the heart (Mjos 1971) The question arises whether or not a part of the increase in MVO observed

tials are due to release of quanta of acetylcholine (ACh) from Schwann cells which after degeneration of the axonal terminals are the only cells lying in close contact with the post synaptic membrane of the muscle fibres (Birks Katz and Miledi 1960)

It was of interest to see whether the release of ACh from Schwann cells was genetically induced in connexion with the degeneration of the nerve terminal For an investigation of this possibility, it was decided to find out whether the min e p p activity of the denervated muscle could be suppressed by actinomycin D (AM) an agent which by blocking the DNA dependent RNA synthesis may prevent the expression of operational gene loci Accordingly AM was given at various times (in a single dose of 75 μg I P) to frogs whose sartorius muscle had been denervated by section of the sciatic nerve Subsequently the muscles were examined for min e p p activity

The effect of AM on this activity was as follows 1) AM almost completely prevented the occurrence of Schwann-cell min e p p s if given the days prior to the time when normally these potentials make their first appearance 2) If given after the Schwann cell min e p p activity had already become established the antibiotic progressively reduced and eventuelly abolished this activity Since it could be shown that under these circumstances the Schwann cells remained in their pre synaptic position and the post synaptic membrane retained its normal ACh sensitivity it follows that AM suppresses the min e p p activity by preventing and reducing the quantal release of ACh from Schwann cells

Since it could also be shown that AM produced a quick and severe suppression of RNA synthesis without at the same time appreciably affecting the translational step of protein synthesis it appears that the quantal release of ACh from Schwann cells depends on the synthesis of an unstable type of RNA Blocking this synthesis just prior to the normal debut of the Schwann-cell min e p p activity results in the failure of this activity to develop at all This suggests that it may be induced in connexion with the nerve degeneration by activation of the Schwann cell s gene locus for control of formation and liberation of ACh

Reference

BIRKS R B KATZ and R MILEDI *J Physiol* (Lond) 1960 *150* 145—168

C 21

Unit Activation in the Post Central Gyrus of the Monkey via Ventral Spinal Pathways

By S A. ANDERSSON and U NORRSELL *Department of Physiology University of Goteborg Sweden*

In the last years the discussion regarding spinal pathways projecting to the cerebral somatosensory areas has dealt mainly with two pathways located in the dorsal half of the spinal cord *the dorsal column pathway and the spino-cervical tract* which forms the spinal link of the spino cervico thalamo-cortical pathway of Morin (1955)

fluence certain physiological characteristics of the nerve terminals which synapse upon it

In the proximal accessory flexor muscle in walking legs of the lobster synaptic potentials in different muscle fibers show markedly different amounts of facilitation on repetitive stimulation even though all the fibers are innervated by a single excitatory axon (Wiersma and Ripley 1952) Potentials in some fibers do not facilitate at all, while in others the potentials increase in size by 20 to 30 times during stimulation at 20 Hz For any single muscle fiber, however stimulation of distinct widely separated groups of synapses produce synaptic potentials with closely correlated facilitation properties The synapses on a single fiber, although made by different branches of the motor axon, thus form a homogenous population with respect to their facilitation properties

The different facilitation characteristics observed in different muscle fibers are not produced by differences among the postsynaptic membrane characteristics of the fibers Quantal analysis of synaptic potentials shows that facilitation can be completely accounted for by an increase in the number of quanta released from the nerve terminals not by an increase in postsynaptic membrane sensitivity Nor is facilitation of transmitter release obscured at poorly facilitating synapses by some type of postsynaptic saturation When Cs is substituted for K in the bathing solution transmitter release and the postsynaptic response increase with no change in the sensitivity of the postsynaptic membrane Since postsynaptic membranes can respond to greater amounts of transmitter substance by producing larger synaptic potentials it is unlikely that these membranes are completely saturated under normal conditions Therefore differences in facilitation properties of synapses reflect real differences in the properties of the presynaptic nerve terminals

These experiments imply that all the presynaptic terminals on a single muscle fiber have matching facilitation characteristics some property of a muscle fiber thus appears to be responsible for the appropriate matching of its own nerve terminals One explanation of this phenomenon is that the nerve terminals are trophically influenced by the muscle fiber that they innervate The mechanism underlying this action of muscle back on nerve is unknown

Reference

WIERSMA C A C and S H RIPLEY *Physiologica Comparata et Oecologia* 195? ? 391—405

C 20

The Suppression of Transmitter Release from Schwann Cells by Actinomycin D

By S BEVAN W GRAMPP and R MILEDI *Department of Biophysics University College London England and Department of Physiology and Medical Physics University of Lund Sweden*

In skeletal muscle of frog following denervation spontaneous miniature end plate potentials (min e p p s) reappear after 2—3 days of electrical silence These poten

transport of 3 hydroxybutyrate and acetoacetate occurs by simple or facilitated diffusion and also whether the transport capacity in the brain increases as fasting progresses

The experiments were performed on normal fed rats and on starved rats on successive days during a 5 days period of starvation The ability of the solutes to pass the blood brain barrier was estimated by means of Oldendorf's brain uptake technique (1970) where the amount taken up by the brain after a single intracarotid injection is expressed relative to the uptake of simultaneously injected tritiated water (which diffuses rapidly into brain) The test substance is labelled with ^{14}C and the results are expressed as the ratio between the tracers in the brain tissue ^{14}C/Tritium (in per cent) the so-called Brain Uptake Index (BUI) The animal is decapitated 15 s after the intracarotid injection and the brain removed for determination of radioactivity

It was found that with increasing concentrations in the injectate the BUI decreased The BUI of 3 hydroxybutyrate fell from 7 9 % at 0 2 mM to 3 3 % at 10 mM concentration in the injectate This observation suggests that the transport is not of a purely diffusional nature Of special interest was the observation that the BUI at a given concentration rose as fasting progressed The BUI of 3 hydroxybutyrate went up from 7 9 % to 11 5 % after 5 days of starvation that of acetoacetate rose from 11 3 % to 17 1 % The differences are significant ($p < 0\ 0125$)

We conclude that the transport of acetoacetate and of 3 hydroxybutyrate across the blood brain barrier probably occurs by facilitated diffusion and that this carrier system shows adaptation during fasting

References

GOTTSTEIN U K HELD W MULLER and W BERGHOFF In Research on the cerebral circulation Charles C Thomas, Publ 1970
HAWKINS R A D H WILLIAMSON and H A KREBS *Biochem J* 1971 *122* 13—18
OLDENDORF W H *Brain Research* 1970 *24* 372—376
OWEN O E A P MORGAN H G KEMP J M SULLIVAN M G HERRERA and G F CAHILL JR *J Clin Invest* 1967 *46* 1589—1595

C 23

On the Pathogenesis of the Intestinal Mucosal Lesions in Shock

By *U* HAGLUND O LUNDGREN and J SVANVIK *Department of Physiology University of Goteborg Sweden*

Hemorrhagic mucosal lesions in the small intestine have been described following impaired intestinal blood flow and shock in rat dog cat and also in man It has been suggested that the lesions located at the tips of the villi are caused by a reduced villous blood flow due to hypotension and/or to intense nervous vasoconstriction (Lillehei *et al* 1967)

Using an indicator dilution technique to record blood flow exclusively in the cat villi it was however demonstrated that blood flow through the superficial layers of the intestinal mucosa is unaltered when perfusion pressure is lowered from 100 mm

The basis of the discussion has been findings made in the carnivora notably the cat Recent experiments have indicated that a different condition prevails in the primate It has thus been found that the cortical projection indicated by short latency initially positive evoked potentials recorded in the post central gyrus of the monkey depends except for the dorsal column on a pathway located in the ventral spinal quadrant contralateral to the afferent input (Andersson, Norrsell and Norrsell 1972) Morin s pathway appears to be present in the monkey but of minor importance (*loc cit*)

Microelectrode recordings now have been made in the arm area of the post central gyrus in the monkey (*M fascicularis*) after transection of the dorsal spinal half at the cervical level C4—C5 The animals were anesthetized with either pentobarbitone sodium or chloralose The units which have been found so far have mostly been modality specific and have required different types of stimulation ranging from hair movement caused by gentle blowing to squeezing and pinching The units requiring gentle peripheral stimuli form a large part of the material The areas of innervation of the individual units range from 0 5 cm² to half of the forearm The latency of activation is when shortest, within the range of the initial positivity of the evoked potential recorded in the intact animal *No surround inhibition has been found yet*

Andersson (1962) and Levitt and Levitt (1968) who made comparable recordings under similar conditions in the cat found no units which could be activated after short latency by gentle peripheral stimuli modality specific or not The present findings thus support the suggestion of Andersson *et al* (1972) that the monkey in contrast to the cat receives somatosensory cortical projection via pathways located in the ventral half of the spinal cord

References

ANDERSSON S A *Acta physiol scand* 1962 56 Suppl 194 1—74
ANDERSSON S A K NORRSELL and U NORRSELL *J Physiol* (Lond) 1972 In press
LEVITT M and J LEVITT *Expl Neurol* 1968 22 276—302
MORIN F *Am J Physiol* 1955 *183* 245—252

C 22

Blood brain Transport Kinetics of Ketone Bodies

By C CRONE and A GJEDDE *Institute of Medical Physiology Dept A University of Copenhagen Denmark*

Interest in blood brain transport of ketone bodies arose from recent publications indicating a significant brain utilization of 3 hydroxyburate and acetoacetate during fasting and ketonaemia (Owen *et al* 1967 Gottstein *et al* 1970 Hawkins *et al* 1971)

Most polar solutes only pass slowly into the brain Ketone bodies may therefore belong to a class of utilizable substrates which despite their polar nature do pass the blood brain barrier at significant rates (like D glucose and some amino acids) Such solutes seem to pass the blood brain barrier by a facilitated diffusion mechanism probably involving an enzymatic step We therefore investigated whether the

transport of 3 hydroxybutyrate and acetoacetate occurs by simple or facilitated dif fusion and also whether the transport capacity in the brain increases as fasting progresses

The experiments were performed on normal fed rats and on starved rats on successive days during a 5 days period of starvation The ability of the solutes to pass the blood brain barrier was estimated by means of Oldendorf's brain uptake tech nique (1970) where the amount taken up by the brain after a single intracarotid injection is expressed relative to the uptake of simultaneously injected tritiated water (which diffuses rapidly into brain) The test substance is labelled with ^{14}C and the results are expressed as the ratio between the tracers in the brain tissue, ^{14}C/Tritium (in per cent) the so-called Brain Uptake Index (BUI) The animal is decapitated 15 s after the intracarotid injection and the brain removed for determination of radioactivity

It was found that with increasing concentrations in the injectate the BUI de creased The BUI of 3 hydroxybutyrate fell from 7 9 % at 0 2 mM to 3 3 % at 10 mM concentration in the injectate This observation suggests that the transport is not of a purely diffusional nature Of special interest was the observation that the BUI at a given concentration rose as fasting progressed The BUI of 3 hydroxybutyrate went up from 7 9 % to 11 5 % after 5 days of starvation that of acetoacetate rose from 11 3 % to 17 1 % The differences are significant ($p < 0\,0125$)

We conclude that the transport of acetoacetate and of 3 hydroxybutyrate across the blood brain barrier probably occurs by facilitated diffusion and that this carrier system shows adaptation during fasting

References

GOTTSTEIN U K HELD W MULLER and W BERGHOFF In Research on the cerebral circu lation Charles C Thomas Publ 1970

HAWKINS R A D H WILLIAMSON and H A KREBS *Biochem J* 1971 *122* 13—18

OLDENDORF W H *Brain Research* 1970 *24* 372—376

OWEN O E A P MORGAN H G KEMP J M SULLIVAN M G HERRERA and G F CAHILL JR *J Clin Invest* 1967 *46* 1589—1595

C 23

On the Pathogenesis of the Intestinal Mucosal Lesions in Shock

By U HAGLUND O LUNDGREN and J SVANVIK *Department of Physiology University of Goteborg Sweden*

Hemorrhagic mucosal lesions in the small intestine have been described following impaired intestinal blood flow and shock in rat dog cat and also in man It has been suggested that the lesions located at the tips of the villi are caused by a reduced villous blood flow due to hypotension and/or to intense nervous vaso constriction (Lillehei *et al* 1967)

Using an indicator dilution technique to record blood flow exclusively in the cat villi it was however demonstrated that blood flow through the superficial layers of the intestinal mucosa is unaltered when perfusion pressure is lowered from 100 mm

Hg to about 40 mm Hg Furthermore intense activation (8 imp/s) of the regional sympathetic vasoconstrictor fibres does not reduce villous blood flow It was also demonstrated that total intestinal blood flow of cats returns almost to control level at the end of 2—2 5 h period of hemorrhagic hypotension at 40 mm Hg despite intermittent vasoconstrictor activation and yet mucosal lesions regularly develop Furthermore intestinal capillary exchange surface area is increased above control throughout the hypotension Thus, it seems less likely that the lesions are caused by a reduced villous blod flow *per se*

The indicator dilution technique revealed however that *mean transit time through the hairpin vascular loops of the villi* is increased from 5—7 s at normal *perfusion pressure* to 20—30 s during hypotension It is suggested that the intestinal *mucosal countercurrent exchanger* becomes for such reasons more efficient during hypotension, greatly increasing the chances for extravascular shortcircuiting of oxygen at the base of the villi Villous tissue hypoxia ultimately leading to the observed mucosal lesions would then develop at the villous tips despite an almost unchanged blood flow in the villi

Reference

LILLEHEI R C R H DIETZMAN and S MOVSAS *Gastroenterology* 1967 52 468—471

C 24

Effects of Ionic Concentration on Permeability Properties of Myelinated Nerve Fibres (Xenopus laevis) Potential Clamp Experiments

By T BRISMAR and B FRANKENHAEUSER *The Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden*

Sodium and potassium are the main current carriers in the nerve membrane (Hodgkin and Huxley 1952, Dodge and Frankenhaeuser 1959) Calcium and magnesium on the other hand seem to have their major effects on the potential dependence of the sodium and potassium permeability systems (Frankenhaeuser 1957 Frankenhaeuser and Hodgkin 1957) The present potential clamp investigation was performed to find out whether changes in [NaCl] and [KCl] have effects on the permeability system *per se* *e g* effects similar to those of calcium

The membrane potential of single nodes of Ranvier in large nerve fibres from *Xenopus laevis* was changed in rectangular pulse steps and the membrane current was measured Curves relating steady state potassium permeability to membrane potential were obtained from current measurements with the fibre in solutions with 2 5 mM KCl and a number of different [NaCl] s and [$CaCl_2$] s Solutions were as far as possible kept isotonic by addition of sucrose

Changes in [NaCl] caused a shift of the steady state potassium permeability potential curve along the potential axis This shift was of positive direction at increased concentration and therefore in the same direction as the *corresponding* shift caused by changes in calcium concentration The shift caused by *sodium* concentration was larger when [Ca] was low than when it was high

Measurements of how the peak sodium permeability to potential relation was affected by solutions with 25 0 mM NaCl and a number of different [KCl] s and [CaCl] s were also made A shift was found which had a corresponding direction, magnitude and calcium dependence

These results may be explained on the basis of the assumptions that negative charges are fixed to the external surface of the membrane (−5 5 μC cm ²) and that the surface potential caused by these charges is affected by the ionic composition of the external solution (see Chandler, Hodgkin and Meves 1965)

References

CHANDLER W K. A L HODGKIN and H MEVES *J Physiol* (Lond) 1965 *180* 821—836
DODGE F A and B FRANKENHAEUSER *J Physiol* (Lond) 1959 *148* 188—200
FRANKENHAEUSER B *J Physiol* (Lond) 1957 *137* 245—260
FRANKENHAEUSER B and A L HODGKIN *J Physiol* (Lond) 1957 *137* 218—244
HODGKIN A L. and A F HUXLEY *J Physiol* (Lond) 1952 *116* 449—472

C 25

An Attempt to Elicit Salivary Secretion by Changing the Intracellular Sodium and Potassium Concentrations Without Applying Neurotransmitters

By J HEDEMARK POULSEN *Institute of Medical Physiology A University of Copenhagen Denmark*

When a salivary gland is stimulated to secrete intracellular potassium is released to the blood (or perfusion fluid) by a passive process (Petersen 1970) The potassium release is associated with passive uptake of an equal amount of sodium (Poulsen 1971) It is possible that the resulting high level of intracellular sodium directly stimulates the active transport mechanisms responsible for the formation of the saliva (Petersen 1971 Poulsen 1971) According to this hypothesis it should be possible to evoke salivary secretion by increasing the intracellular sodium concentration provided that the transport mechanisms forming the saliva are intact

Cat submandibular glands were perfused with standard Locke solution and K free Locke solution The temperature was kept at 37° C or reduced to 5° C when stated. The potassium concentration of the venous perfusion fluid and the amount of saliva ecreted were measured When the temperature of the gland was reduced to 5° C intracellular potassium was lost to the perfusion fluid This potassium loss was probably accompanied by sodium uptake Rewarming the gland always caused a pronounced uptake of potassium (and probably extrusion of sodium) while salivary secretion was never evoked

During perfusion with K free Locke solution a marked potassium loss was also observed Again potassium loss was undoubtedly associated with sodium uptake However salivary secretion was never elicited by K free perfusion nor by re introduction of standard Locke solution which caused an active uptake of potassium During the K free period it was possible to evoke salivary secretion by acetylcholine

The intracellular sodium concentration of the cat submandibular gland has thus been increased by inhibiting the ordinary sodium potssium pump by low tempera

ture or by low extracellular potassium concentration Neither of these situations nor the return to normal temperature and normal extracellular potassium concentration did evoke salivary secretion These findings seem to be incompatible with a direct activating effect of the intracellular sodium concentration on the secretory mechanisms

References

PETERSEN O H *J Physiol* 1970 *208* 431—447
PETERSEN O H *Phil Trans Roy Soc Lond B* 1971 *262* 307—314
POULSEN *J H Proc Internat Union Physiol Sci* 1971 IX 240

C 26

Isovolumetric Capillary Pressure and Compliance Characteristics of the Interstitial Fluid Space in Skeletal Muscle

By E ELIASSEN, B FOLKOW S HILTON and B ÖBERG *Department of Physiology University of Goteborg Sweden*

Experiments were performed to determine the isovolumetric capillary pressure level (P_{CI}) in the calf muscles of the cat and to examine the pressure volume relationship of the interstitial space in this tissue A high compliance of this latter compartment would seem to be a prerequisite for an easy exchange of fluid betwen blood and the muscle extravascular space Guyton (1965) however suggests that this compliance is low in the normal tissue pressure range

The calf was isolated and placed in a plethysmograph for continuous recording of tissue volume changes The preparation was either perfused in the normal way by the cat s own blod or intermittently by oxygenated Tyrode solution containing 4 % or 6 % Dextran (Macrodex) Venous outflow was measured by a drop recorder Arterial inflow pressure (P_A) and venous outflow pressure (P_V) were recorded and could be set at desired levels By appropriate resettings of P_A and P_V the capillary pressure (P_C) could be adjusted so that an isovolumetric state or a filtration or an absorption of fluid could be established P_C was measured by a modification of the technique described by Pappenheimer and Soto-Rivera (1948) To prevent autoregulatory adjustments of resistance and P_C during the experimental procedures most measurements were performed with the vessels maximally dilated caused by administration of large amounts of papaverine or isoprenaline

P_C in the normal control situation amounted to 12—14 mm Hg at a calculated plasma colloid osmotic pressure around 19—23 mm Hg Lowering P_C or shifting to 6 % Dextran perfusion led to a fluid absorption by which the interstitial fluid volume (IFV) could be reduced 30 % below control (assumed to comprise 15 % of total muscle weight) The concomitant decrease of P_C, then amounted to only 4—5 mm Hg Conversely an increase of IFV by 100—200 % above control caused by inducing a net fluid filtration led to a P_{CI} rise of only some 3—5 mm Hg The shifts in tissue pressure with these large variations of IFV must according to the Starling concept be still smaller since the interstitial colloid osmotic pressure must rise during fluid absorption and fall during filtration It is therefore concluded that the com

pliance of the muscle interstitial compartment is high and that large variations of IFV can be produced even by small alterations of P_C

References

GUYTON A *Circulat Res* 1965 *16* 452—460
PAPPENHEIMER, J R. and A SOTO-RIVERA *Amer J Physiol* 1948 *152* 471—491

C 27

Isovolumetric Capillary Pressure during Rest and Sympathetic Nerve Activity in Canine Subcutaneous Adipose Tissue

By M INTAGLIETTA and S ROSELL *Department of Pharmacology Karolinska Institutet Stockholm*

Fredholm Öberg and Rosell (1970) postulated that activity of sympathetic nerves to canine subcutaneous adipose tissue may increase vascular permeability This suggestion was supported by the findings that (1) sympathetic nerve activity induces a pronounced increase in the capillary filtration coefficient (CFC) despite a concomitant vasoconstriction and (2) that the permeability—surface area product (PS) was reduced There is thus an apparent discrepancy between the results from filtration (CFC) and diffusion (PS) measurements To explain this the hypothesis was advanced that sympathetic nerve activity diminishes the number of patent capillaries concomitantly with an increase in permeability

The present study was undertaken with the idea that a higher capillary permeability might affect the isovolumetric capillary pressure (P) provided that the permeability increase is of such a nature that osmotically active molecules become diffusible across the capillary membrane

Experiments were performed on canine subcutaneous adipose tissue placed in a plethysmograph (Rosell 1966 Öberg and Rosell 1967) Blood flow arterial and venous blood pressure and tissue volume were recorded continuously P was determined according to the principle of Pappenheimer and Soto-Rivera (1948) The blood flow was reduced in a stepwise fashion by means of a screw clamp on the artery The level of the venous pressure was then adjusted to give hydrostatic capillary pressure which rendered the adipose tissue isovolumetric The arterial and venous pressures at isovolumetric states were plotted versus blood flow and P estimated by linear extrapolation to zero pressure difference In all experiments this was found to coincide with zero blood flow The linear relationship between isovolumetric arterial and venous pressures respectively versus blood flow is evidently a consequence of the lack of autoregulatory responses Therefore this method can be used with some precision to determine P_i in adipose tissue

The mean resting isovolumetric capillary pressure was 10 1 mm Hg (range 20 8—3 8 n = 13) Sympathetic nerve stimulation (3—9 Hz 2 msec 7—10 V) reduced P_i which averaged 5 6 mm Hg (range 10 8—0) The reduced P_c is compatible with the hypothesis of an increase in vascular permeability following sympathetic nerve activity

References

FREDHOLM B B ÖBERG and S ROSELL *Acta physiol scand* 1970 79 564—574
PAPPENHEIMER, J R. and A SOTO RIVERA *Am J Physiol* 1948 152 471—491
ROSELL S *Acta physiol scand* 1966 67 343—351
ÖBERG B and S ROSELL *Acta physiol scand* 1967 71 47—56

C 28

Intratubular Carbon Dioxide Tension in the Proximal Tubule of the Kidney

By B KARLMARK *The Institute of Physiology and Medical Biophysics Biomedical Center University of Uppsala Sweden*

Previous micropuncture studies on the cortical nephron of the rat indicate that the hydrogen ions secreted from the proximal tubular cells form carbonic acid together with the bicarbonate ions filtered into the Bowman s capsule The carbonic acid, in turn will decompose cathalyzed or not into water and carbon dioxide which diffuses to the peritubular blood (Rector *et al* 1965) This out diffusion of carbon dioxide is further thought to be an extremely rapid process To test the validity of such an hypothesis that carbon dioxide is instantaneously equilibrated between the lumen and peritubular blood micropuncture experiments were performed on the proximal tubules of the rat cortical nephrons

Primarily the intratubular pH was measured *in vivo* with an antimony micro electrode system Thereafter luminal fluid was anaerobically collected with precautions to prevent the escape of carbon dioxide The samples thus obtained of a few nanoliter size were equilibrated with 3 different carbon dioxide tensions and the pH was measured *in vitro* after each equilibration using similar antimony microelectrodes (Karlmark and Sohtell 1972) The pH/log P_{CO_2} relations were plotted in a diagram yielding the buffer line of that sample The actual pH initially measured *in vivo* applied to this line gave the actual carbon dioxide tension presumed to exist in the luminal fluid *in vivo*

The actual pH values thus measured *in vivo* were found to be higher than those calculated from the buffer line at the carbon dioxide tensions of the arterial blood This can be explained in at least three different ways *a*) A carbon dioxide tension in the peritubular blood which is remarkably higher than that of the arterial blood *b*) A chemical non equilibrium (disequilibrium) intraluminally, yielding excess carbonic acid and thus a lower pH measured *in vivo* as compared to that measured *in vitro* under equilibrium conditions *c*) An intraluminal carbon dioxide tension which is higher than that in the peritubular blood indicating a rather slow diffusion process for carbon dioxide out of the lumen

The present study favours the last explanation as quantitatively dominating

References

KARLMARK B and M SOHTELL *Analyt Biochem* In Press
RECTOR F C N W CARTER and D W SELDIN *J Clin Invest* 1965 44 2 278—290

Acta physiol scand 1973 87 145—157

From the Department of Pharmacology Karolinska Institutet Stockholm Sweden

Interaction of Adenosine 5'-Triphosphate (ATP) with Histamine Release Induced by Compound 48/80

By

RUNE DAHLQUIST BERTIL DIAMANT and KERSTIN ELWIN

Received 21 December 1971

Abstract

DAHLQUIST R B DIAMANT and K ELWIN *Interaction of adenosine 5 triphosphate (ATP) with histamine release induced by compound 48/80* Acta physiol scand 1973 *87* 145—157

The histamine releasing action of compound 48/80 disappeared after pretreatment of rat mast cells with ATP in the absence of divalent cations The inhibitory action was evident already with 2 μM of ATP provided the cells were preincubated with ATP for short periods of time prior to the addition of 48/80 When mast cells were exposed to 48/80 combined with ATP more than 10 μM of ATP was needed to induce inhibition of histamine release This inhibitory effect was found to be specific for ATP compared with various organic phosphorous compounds tested and was not mimicked by EDTA With concentrations of ATP less than 10 μM the sensitivity to the action of 48/80 was spontaneously restored after prolonged preincubation of the cells with ATP The experimental findings suggest that ATP in the absence of divalent cations induced configurational changes of the plasma membrane of the mast cells so that 48/80 could no longer exert its degranulating and histamine releasing action

Histamine release can be induced from isolated rat mast cells by various histamine releasing agents One such agent compared 48/80 causes the extrusion of granules from the cells incubated at 37° C The histamine releasing process occurs within the first 20 s after contact between the releasing agent and the cells (Moran Uvnas and Westerholm 1962 Bloom Fredholm and Haegermark 1967 Sugiyama 1971)

To the contrary when mast cells are incubated with ATP in the presence of Ca^{++} a more prolonged release process develops from the cells Histamine release occurs over several minutes and is accompanied by the extrusion of granules from the cells (Sugiyama 1971 Kruger *et al* to be publ) At 37° C the release is preceded by a lag period of about one minute during which time certain intracellular morphological changes occurs (Diamant and Peterson 1970 Sugiyama 1971 Kruger *et al* to be publ) The minimum concentration of ATP in the medium which releases histamine in the presence of Ca^{++} is of the order 10 M In the absence of Ca^{++} ATP does not induce histamine release or granular extrusion although various intra cellular morphological changes develop such as swelling of the cells and oscillation

of granular material within the cells (Diamant and Kruger 1967 Sugiyama and Yamasaki 1969 Sugiyama 1971, Kruger *et al* to be publ)

It has been demonstrated that, irrespective of the absence or presence of Ca^{++} in concentrations optimal for histamine release (0 25 mM) ATP causes mast cells to swell and sodium as well as trypan blue to accumulate in the cells These effects occur within seconds after mast cells are brought into contact with ATP This indicates that permeability changes are immediately induced due to interaction of ATP with some component of the plasma membrane (Kruger *et al* to be publ Dahlquist *et al* to be publ) Thus even in the absence of Ca when no histamine release occurs ATP seems to induce certain immediate effects on the mast cell membrane

Compound 48/80 is considered to induce degranulation and histamine release from isolated rat mast cells by a process that has no absolute prerequisite for Ca^{++} in the incubation medium (Uvnas and Thon 1961 Saeki 1964) The present investigation was performed in order to investigate whether ATP in the absence of Ca would influence histamine release induced by compound 48/80 A preliminary report on parts of the present investigations has been presented earlier (Diamant and Dahlquist 1970)

Methods and Materials

Isolation of rat mast cells

Mast cells from the abdominal and thoracic cavities of male Sprague Dawley rats (weight 350—450 g) were isolated by centrifugation in a Ficoll density gradient according to the method of Uvnas and Thon (1959) with the minor modification described by Thon and Uvnas in 1966 The mast cells collected from the Ficoll were centrifuged (350×g) and washed three times at room temperature each time with 5 ml of salt solution (containing NaCl 145 mM and KCl 2 7 mM) which was buffered to pH 7 0 with 10 % (v/v) Sorensen phosphate buffer (67 mM) In addition the buffered salt solution contained human serum albumin 1 mg/ml The mast cells were finally resuspended in between 300 and 800 µl of the buffered salt solution giving a final concentration of 1 2—3 6×10⁶ cells/ml The cells were counted in a Burker chamber and contained 90—95 % mast cells

Incubation procedures

Model I Isolated rat mast cells were diluted in 2 ml of buffered salt solution at 37 C giving a final concentration varying in separate experiments between 2—5×10⁴ cells/ml After incubation of mast cells with ATP for various lengths of time compound 48/80 was added to the same tubes The incubation was continued for an additional 10 minutes and was terminated by placing the tubes in an ice bath This incubation procedure was used in the first part of the present study (Fig 1—7 Table I)

Model II To investigate whether the inhibitory effect of ATP on the action of 48/80 might be due to a direct interaction between ATP and compound 48/80 in the incubation medium more concentrated cells (1 5—2 5×10⁵ mast cells/ml) were pretreated with ATP in a smaller volume (100 µl) After various lengths of time 20 µl were removed and added to tubes containing 2 ml of buffered salt solution and eventually compound 48/80 Following 10 minutes at 37 C these tubes were placed in an ice bath This incubation procedure was also employed to investigate the influence of the metabolic inhibitor antimycin A as well as of the temperature on the action of ATP on the cells

After incubation the tubes were centrifuged for 10 minutes at 350×g at 2—4 C The supernatants were decanted into new tubes and " ml of distilled water were added to the cell sediment to extract the remaining histamine

Histamine determination
Histamine was determined by the fluorescence method of Shore *et al* (1959) omitting the extraction procedure in accordance with the observation of Bergendorff (1965) that histamine is the only substance extracted from rat mast cells which reacts to give fluorescence with o-phthalyaldehyde The fluorescence was read in a Farrand fluorometer Model A 2 or a Farrand ratio fluorometer (Farrand Optical Co Ltd USA)

The histamine release was expressed as a percentage appearing in the supernatant of the total histamine content of the cells

The spontaneous release of histamine (i e in the absence of compound 48/80 and ATP) amounted to less than 6 % and has been deducted from all values presented Unless otherwise stated the release obtained by ATP alone in the absence of 48/80 has also been subtracted from the histamine release observed in cell samples exposed to both ATP and 48/80 This ATP induced histamine release amounted to less than 7 % of the total histamine content except where the actual values are shown in the figures or stated in their legends

All samples were run in duplicate

Materials

Human serum albumin was kindly supplied by AB Kabi Stockholm Sweden Compound 48/80 was kindly supplied by AB Leo Helsingborg Sweden Ficoll was purchased from AB Pharmacia Uppsala Sweden Adenosine 5 triphosphate (ATP) (sodium salt) was obtained from Sigma Chemical Comp St Louis USA Stock solution of ATP (about 100 mM) was assayed spectrophotometrically pH was adjusted to 7 by addition of NaOH The stock solution was stored at −20 C Adenosine and L(+) lactic acid (sodium salt) were purchased from Boehringer & Soehne GmbH Mannheim Germany Antimycin A adenosine 3 5 cyclic monophosphoric acid (3 5 AMP) and the sodium salts of the compounds listed below were all obtained from Sigma Chemical Comp (St Louis USA)

Adenosine 5 monophosphate (AMP) N^6 O^2 dibutyryl adenosine 3 5 cyclic monophosphoric acid (dibut 3 5 AMP) adenosine 5-diphosphate (ADP) cytidine 5 triphosphate (CTP) guanosine 5 triphosphate (GTP) inosine 5 triphosphate (ITP) thymidine 5 triphospate (TTP) uridine 5 triphosphate (UTP) D glucose 1 phosphate (G1P) D glucose 6 phosphate (G6P) 6 phosphogluconic acid (6 PG) pyruvic acid phosphoenol pyruvic acid (PEP) phosphocreatine (PCr)

Results

When isolated rat mast cells were added to a Ca^{++} free incubation medium containing 48/80 (0 5 μg/ml) and increasing concentration of ATP (1—100 μM) the histamine release induced by 48/80 was found to be inhibited by the presence of ATP above 10 μM (Fig 1) No inhibition occurred with ATP below 10 μM on the contrary if anything the release was slightly activated

When mast cells were preincubated in a Ca free medium for 2 5 min with ATP (1—10 μM) prior to the adddition of 48/80 (0 6 μg/ml) histamine release induced by 48/80 became markedly inhibited with 2 μM of ATP (Fig 2) When the concentration of ATP exceeded 3 μM during the preincubation period subsequent exposure of the cells to 48/80 did not induce histamine release that exceeded the slight release induced by ATP alone (< 7 % of the total histamine content)

The time course (Fig 3) for the ATP induced inhibition of the histamine release caused by 48/80 revealed that when the cells were preincubated for various lengths of time (up to 60 s) with 6 μM of ATP before they were exposed to 48/80 inhibition gradually occurred during the first 45 s of incubation with ATP at which time the cells no longer responded with histamine release when exposed to 48/80 With 32 μM of ATP partial inhibition was noted when the cells were exposed to 48/80 and ATP simultaneously (time 0 in Fig 3) Already 5 s of preincubation of the cells with ATP totally inhibited subsequent histamine release by 48/80

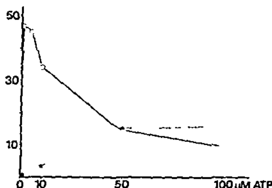


Fig 1

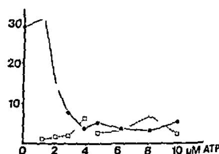


Fig 2

Fig 1 Influence on 48/80 induced histamine release by the simultaneous presence of ATP (1—100 µM) in the incubation medium
○———○ 48/80 (0 4 µg/ml)
✶— — — —✶ control samples without 48/80
The histamine release observed with ATP alone was not subtracted from the release values obtained from samples exposed to the combination of ATP and 48/80

Fig 2 Influence on 48/80 induced histamine release by preincubation of the mast cells for 2 5 min with ATP (1—10 µM) prior to the addition of 48/80
●———● 48/80 (0 6 µg/ml)
□— — — —□ control samples without 48/80
The histamine release observed with ATP alone was not subtracted from the release values obtained from samples exposed to the combination of ATP and 48/80

When mast cells were preincubated with ATP (4—14 µM) for 2 5 and 20 min respectively prior to the addition of 48/80 it was found that higher concentrations of ATP were needed to induce inhibition when the cells had been exposed to ATP for 20 min as compared to 2 5 min (Fig 4) For example with 4 µM of ATP complete inhibition of the histamine releasing activity of 48/80 was observed when he cells had been in contact with ATP for 2 5 min whereas no inhibition was found after exposure of the cells to ATP for 20 min

The experiments indicated that the inhibitory effect of ATP at concentrations less than 10 µM spontaneously disappeared upon prolonged incubation of the cells with ATP prior to the addition of 48/80 The spontaneous reversal of the inhibitory effect of ATP on 48/80 induced histamine release upon prolonged incubation is further evident from Fig 5 and Fig 6 When rat mast cells were preincubated for 30 s with ATP (5 8 or 30 µM Fig 5) a pronounced inhibition of the histamine release induced by 48/80 was observed Complete inhibition was found with all three concentrations of ATP after 2 5 min of preincubation After prolonged incubation with ATP (10 and 20 min) the inhibition was maintained with the highest concentration of ATP whereas with the two lower concentrations the inhibition spontaneously disappeared When ATP (5 and 8 µM) together with 48/80 was added to the cells an activation of the release was found whereas with 30 µM of ATP slight inhibition was noted as compared to the release induced by 48/80 alone

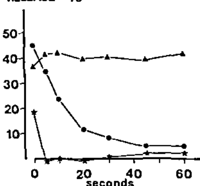


Fig 3

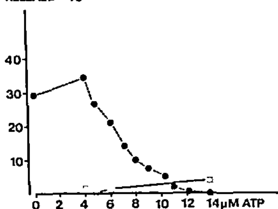


Fig 4

Fig 3 Time course for ATP induced inhibition of the histamine release caused by 48/80 Abscissa Length of preincubation time (seconds) of mast cells with ATP prior to the addition of 48/80 (0 7 μg/ml) The shortest time investigated was 5 s Zero time denotes the addition of mast cells to a mixture of ATP and 48/80

★———★ ATP 32 μM

●———● ATP 6 μM

▲———▲ control samples not pretreated with ATP

Fig 4 Inhibition of 48/80 induced histamine relea e caused by pretreatment of the cells with ATP (4—14 μM) for 2 5 min (□——□) and 20 min (●——●) prior to the addition of 48/80 (0 6 μg/ml)

In Fig 6 eleven similarly performed experiments are presented The results show that preincubation of mast cells with ATP (4—5 μM) exerted an inhibitory effect on subsequent histamine release induced by the addition of 48/80 (0 6 μg/ml) and that this inhibition was dependent on the time the cells were preincubated with ATP Preincubation for 2 5 min with ATP induced complete inhibition Preincubation for 10 min showed inhibition although less than after 2 5 min and preincubation for 20 min showed that the cells responded to 48/80 with histamine release that did not significantly differ from controls without ATP The simultaneous addition of cells to a mixture of ATP and 48/80 caused a slight activation of the histamine release which however in this series of experiments was not statistically significant

The spontaneous restoration of the sensitivity of mast cells towards 48/80 after exposure to ATP (< 10 μM) for prolonged periods of time might be ascribed to the possibility that ATP gradually decreased in the incubation medium to levels that were no longer inhibitory possibly due to the action of an ATP ase in the plasma membrane of mast cells (Diamant 1967 Coffey 1970 or of contaminating cells of non mast cell origin (Sugiuama 1971 a) To investigate this possibility rat mast cells were exposed to ATP (6 μM) for 1 and 10 min prior to the addition of 48/80 (0 6 μg/ml) In order to achieve optimum restoration of the sensitivity of the cells to 48/80 glucose (5.6×10^{-4} M) was added (Dahlquist *et al* 1973 a) As can be noted from Fig 7 inhibition of the action of 48/80 was pronounced after exposure of the

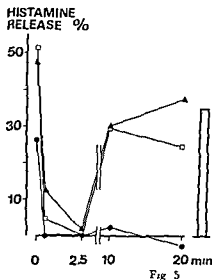


Fig 5

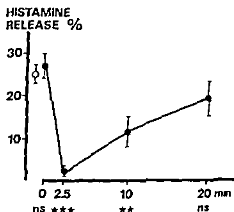


Fig 6

Fig 5 Time course for development and cessation of the inhibition of 48/80 induced histamine release caused by pretreatment of mast cells with different concentrations of ATP prior to the addition of 48/80 (0 6 ug/ml) Abscissa Preincubation time (min) of mast cells with ATP before the addition of 48/80

● ——— ● ATP 30 μM
□ ——— □ ATP 8 μM
▲ ——— ▲ ATP 5 μM

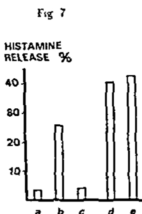
The column represent 48/80 induced histamine release from control cells not exposed to ATP The histamine release induced by 30 μM of ATP alone was 8 % at the 10 min point (20 min of incubation) and 10 % at the 20 min point (30 min of incubation)

Fig 6 Influence of the preincubation time of mast cells with ATP (4—5 μM) on the histamine release induced by the subsequent addition of 48/80 (0 6 ug/ml) Zero time denotes the addition of mast cells to a mixture of ATP and compound 48/80 The unfilled circle represents 48/80 induced histamine release from control cells not exposed to ATP

Mean and S E of 11 expts The degree of significance of the differences between ATP treated means and control mean is given below the abscissa

cells to ATP for 1 min (a) and the sensitivity was partially restored after 10 min of preincubation of the cells with ATP (b) In a parallel sample the cells were centrifuged after having been incubated with ATP for 10 min and new cells were incubated with the supernatant After incubation of these new cells for 1 min 48/80 was added (c) The results show that these new cells had become equally intensitive towards the action of 48/80 as those during the first incubation It could therefore be concluded that the restoration of the sensitivity of the mast cells towards 48/80 after prolonged incubation with ATP was not due to ATP decreasing to non effective concentrations

In order to investigate whether the inhibition of 48/80 induced histamine release caused by preincubation of mast cells with ATP could be elicited by other organic phosphorous compounds or metabolic intermediates such substances (see Table I Exp A and B) were incubated in a concentration of 10^{-4} M with mast cells for 5 min at 37° C prior to the addition of 48/80 (0 6 ug/ml) Dibutyryl 3′ 5 AMP (Table I Exp C) was preincubated with the cells in a concentration of 5×10^{-4} M



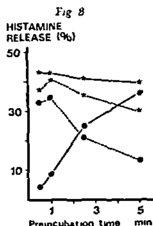


Fig 7 A biological assay of ATP in the incubation medium after 10 min of contact between ATP and the mast cells a Inhibition of 48/80 induced histamine release by pretreatment of the cells with ATP for 1 min prior to the addition of 48/80 (Control sample not treated with ATP is represented by column d) b Restoration of the sensitivity of the mast cells with regard to histamine release induced by 48/80 after 10 min of contact between ATP and the cells before the addition of 48/80 c Inhibition of 48/80 induced histamine release from ne cells preincubated for 1 min with the supernatant of a parallel sample to b prior to the addition of 48/80 (Control sample not treated with ATP is represented by column e)

The concentrations of ATP and 48/80 used in this experiment was 6 μM and 0 6 μg/ml respectively Glucose 5 6 × 10^{-4} M was present in the incubation medium

The histamine release in control samples treated with ATP and glucose in the absence of 48/80 amounted to 9 13 and 10 % in samples corresponding to column a b and c respectively

Fig 8 Influence of low temperature on the rate of development of the inhibition of 48/80 induced histamine release caused by ATP Concentrated cell suspensions (see Methods section) were preincubated with ATP (34 μM) for various lengths of time (denoted on the abscissa) Cell samples were then transferred to 2 ml of buffered salt solution at 37 C containing 48/80 (0 6 μg/ml) Glucose 5 6 × 10^{-4} M was present during the preincubation

Symbols

● ——— ● Mast cells preincubated with ATP at 37 C prior to dilution and exposure to 48/80

● – – – – ● Mast cells preincubated with ATP at 0 C prior to dilution and exposure to 48/80

★ ——— ★ Mast cells preincubated at 37 C in the absence of ATP prior to dilution and exposure to 48/80

★ – – – – ★ Mast cell preincubated at 0 C in the absence of ATP prior to dilution and exposure to 48/80

The histamine release in control samples treated with ATP and glucose in the absence of 48/80 for 5 min at 37 C amounted to 9 % Mean of 2 expts

for 2 5 min prior to the addition of 48/80 As evident from the table (Exp A—C) none of the compounds tested induced histamine release by themselves that exceeded the spontaneous histamine release (4 %) ATP by itself released 7—10 % of the total histamine content above the spontaneous release

With the exception of ATP none of the compounds tested inhibited 48/80 induced histamine release under the experimental procedures used In addition CTP in both experiments as well as dibutyryl 3 5 AMP (Table I Exp A—C) enhanced 48/80 induced histamine release by an additional 13—18 % of the total histamine content of the cells in comparison with 48/80 alone This effect of CTP and dibutyryl 3 5 AMP was not further investigated as to statistical significance or mechanism of action

TABLE I Histamine release induced by 48/80 (0 6 µg/ml) after pretreatment of the mast cells with the compounds listed For interpretation of abbreviations see the section of methods and materials

	Pretreatment time with each compound prior to the addition of 48/80
Expts A and B	5 min
Exp C	2 5 min
Exp D	10 min
Expts E F and G	10 min

Exp A Compound (10^{-4} M)	Histamine release (%) No 48/80	48/80
—	4	35
ATP	11	3
GTP	3	35
UTP	3	36
TTP	3	5
CTP	3	33
ITP	4	36
AMP	3	35
PCr	4	36
FEP	4	30
G6P	3	33
Adenosine	5	34

ADP (10^{-6} M—2×10^{-4} M) did not inhibit histamine release induced by the subsequent exposure of the cells to 48/80 (Table I Exp D) By increasing the concentration of ADP above 10^{-4} M however inhibition of 48/80 induced histamine release occurred This was attributed to ATP contaminating the ADP preparation (compare Diamant and Kruger 1967)

Since ATP might be considered to exert its inhibitory action by chelating cations, the effect of EDTA was investigated (Table I Exp E F and G) EDTA (1 mM) was found not to influence histamine release induced by 48/80 in a Ca^{++} free incubation medium Neither did EDTA induce histamine release by itself Thus among the compounds tested the inhibitory effect on 48/80 induced histamine release was found to be specific for ATP and was not evident with other organic triphosphates adenosine compounds glycolytic intermediary products or EDTA

In the experiments so far presented, mast cells were incubated with ATP in 2 ml of a Ca free buffer solution (final concentration 2—5×10^{5} cells/ml) and 48/80 was added to the same tubes In the following experiments the mast cells were preincubated with ATP and eventually antimycin A in 100 µl of Ca free buffer solution (final concentration $1\,5$—$2\,5\times10$ cells/ml) After the preincubation period 20 µl of the cell suspension were transferred to 2 ml of buffer solution at 37° C

TABELL I (continued)

Exp B Compound (10 M)	Histamine release ( ) No 48/80	48/80
—	4	39
ATP	14	−1
CTP	5	55
Lactate	5	40
Pyruvate	4	40
CIP	6	39
6 PG	5	35
AMP	5	40
3 5 AMP	6	38

Exp C Compound (5×10 M)	Histamine release ( ) No 48 80	48/80
—	4	28
dibut 3 5 AMP	6	41
Exp D Compound (10 M 2×10 M)	Histamine release ( ) No 48/80	48/80
—	1	55
ADP	2−4	51−58

Expts E F and G Compound (10 M)	Histamine release ( ) No 48/80	48/80
—	4 (3−6)	28 (25−31)
EDTA	6 (5−8)	31 (28−36)
Mean and range		

thereby diluting ATP (and when present antimycin A) 100 times to non effective concentrations before the cells were exposed to 48/80

Histamine release induced by compound 48/80 is completely blocked by antimycin A (10^{-7} M) provided glucose is omitted from the incubation medium This inhibition disappears after a few min following dilution of the cell suspensions to ineffective concentrations of the inhibitor prior to the addition of 48/80 (Diamant and Peterson unpublished observations) In order to investigate whether antimycin A would influence the inhibition exerted by ATP on the action of 48/80 on mast cells concentrated mast cells were preincubated with antimycin A (10^{-7} M) for

5 min ATP (35 μM) was added and 30 s later the cell suspension was diluted 100 times into new buffer solution at 37° C Five min thereafter 48/80 (0 6 μg/ml) was added and histamine release was determined after an additional 10 min of incubation It was found that control cells (incubated in the absence of ATP and antimycin A) released 45 % of their histamine and antimycin A treated cells 41 % ATP treated cells released only 3 %, and cells exposed to antimycin A as well as to ATP, released 2 % of their histamine It can therefore be concluded that antimycin A did not influence the inhibition of 48/80 induced histamine release exerted by ATP

The inhibition induced by ATP on the action of 48/80 was found to be dependent on temperature (Fig 8) When concentrated mast cells were preincubated with ATP (34 μM) and glucose (5 6×10⁴ M) at 0° C and cell samples were transferred after various times of preincubation to 2 ml of 48/80 containing buffer solution at 37° C the inhibition was not apparent until after between 1 and 2 5 min of preincubation and further increased after 5 min to exposure of the cells to ATP In comparison it can be noted that cells incubated similarly at 37° C showed maximal inhibition after 30 s of contact with ATP and that the sensitivity was gradually and totally restored after prolonged preincubation (2 5 and 5 min)

Thus the inhibition of the action of 48/80 caused by ATP developed slower at 0° C as compared to 37° C

The restoration of the sensitivity of the cells towards the action of 48/80 observed in the present experiment after prolonged preincubation of the cells even with 34 μM of ATP was due partly to the fact that the cells during the preincubation periods were comparatively concentrated (1 5—2 5×10 cells/ml) and partly to the presence of glucose (Dahlquist *et al* 1973 a) The experiment further suggests that the inhibitory action of ATP on the cells after 30 s of preincubation was not due to an extracellular binding between 48/80 and ATP whereby 48/80 might be come inactivated

Discussion

It is evident from the present investigation that in the absence of divalent cations ATP exerted a specific action on rat mast cells whereby the cells became insensitive towards the action of compound 48/80 Provided the cells were preincubated with ATP for short periods of time prior to the addition of 48/80 the inhibitory action was evident already with 2—3 μM of ATP thus markedly lower concentrations than ever in our hands have been found to induce histamine release from mast cells The time course by which the inhibition developed was dose dependent and occurred within 5 s under suitable conditions When mast cells were exposed to 48/80 and ATP simultaneously more than 10 μM of ATP was need to inhibit the histamine releasing action of 48/80 Since histamine release from rat mast cells induced by 48/80 at 37° C is completed within 20 s (Moran Uvnas and Westerholm 1962 Bloom, Fredholm and Haegermark 1967 Sugiyama 1971) it is apparent that when

mast cells were exposed to a mixture of 48/80 and ATP ATP exerted its action on the cell before degranulation and histamine release was initiated by 48/80 From the morphological point of view it has been established that ATP inhibits the de granulating action of 48/80 (Diamant and Kruger unpublished observations)

The mechanism by which ATP inhibited the action of 48/80 seems according to our present knowledge to be via an effect on the plasma membrane of the mast cells In favour of this view the observations are that ATP induces a rapid uptake of sodium and a loss of potassium from the cells as well as an uptake of trypan blue to the cell nucleus (maximal effects within 60 s) (Dahlquist *et al* to be publ Kruger *et al* to be publ) Such ATP induced changes of the membrane permeability seems likely to be related to the configuration changes noted on mast cells after local application of ATP to the cell membrane (Diamant and Kruger 1968) as well as to ATP induced swelling of the mast cells as was reported in an electronmicroscopic study (Bloom *et al* 1970) Recently this swelling phenomenon was reported to occur with a half maximal effect within 15 s when mast cells were incubated with ATP (10^{-4} M) in the absence of Ca^{++} (Sugiyama 1971)

In the same report it was demonstrated that already 5×10^{-6} M of ATP caused the diameter of mast cells to increase from 12 to 17 μ and maximum swelling (20 μ) was observed with 10^{-5} M of ATP Thus the concentration of ATP which will in duce swelling of the cells and inhibition of histamine release caused by 48/80 seems to be of the same order Both cellular effects occur at lower concentrations of ATP than needed to induce histamine release from mast cells in the presence of Ca

The velocity by which ATP exerted its inhibitory effect on the action of 48/80 was markedly decreased at 0° C The inhibition developed however but was in complete even after pretreatment of the cells with ATP for 5 min whereas at 37° C 30 s of pretreatment was sufficient to induce complete inhibition of the action of 48/80

In addition it was found that cells treated with antimycin A were still sensitive to the action of ATP Thus the inhibition exerted by ATP did not seem to depend on an intact oxidative metabolism of the mast cells in contrast to the histamine releasing effect induced by ATP on the cells in the presence of Ca (Diamant and Peterson 1971) In agreement with the present findings are the observations that various metabolic inhibitors including some active on oxidative phosphorylation did not influence the swelling phenomenon of mast cells induced by ATP (Sugiyama 1971)

The possibility that ATP might exert its inhibitory action by chelating cations in the plasma membrane necessary for maintaining an intact and responsive membrane for the action of 48/80 seems presently unlikely since EDTA did not mimic the effect of ATP Histamine release in the absence of added Ca occurred equally well by 48/80 in the presence of EDTA as in its absence

The present investigation has further revealed that the inhibitory action of ATP on the sensitivity of mast cells towards 48/80 would under suitable experimental conditions disappear upon prolonged incubation of the cells with ATP prior to the

addition of 48/80 The disappearance of the inhibitory effect of ATP was dose dependent and was shown not to be due to a gradual loss of ATP in the cell suspensions to ineffective levels following prolonged incubation In diluted cell suspensions (2—5×10³ cell/ml) no restoration of the sensitivity of the cells towards 48/80 was observed even after prolonged preincubation of the cells (up to 20 min) with ATP above 10 μM

A likely mechanism of action for ATP to inhibit the degranulation and histamine releasing effect of 48/80 on the mast cell would be that ATP induced changes of the plasma membrane The involvement of the plasma membrane is indicated by changes of fluxes of cations, and swelling of the cell The changes of the plasma membrane most likely would involve disturbances of the molecular configuration and changes of electrical charges Hereby the binding sites for 48/80 in the plasma membrane might be altered so that they no longer could react with the compound Therefore degranulation and histamine release no longer would be induced by 48/80 The findings indicate that at least with low concentrations of ATP the changes of the plasma membrane are spontaneously reversible upon prolonged incubation

This study was supported by the Swedish Medical Research Council (grant nr B71 14X 1004 06A) Karolinska Institutet Stockholm Sweden and Hesselmans Research Foundation Sweden

References

BERGENDORFF A Unpublished observation 1965

BLOOM G D B FREDHOLM and Ö HAEGERMARK Studies on the time course of histamine release and morphological changes induced by histamine liberators in rat peritoneal mast cells *Acta physiol scand* 1967 *71* 270—282

BLOOM G D B DIAMANT Ö HAEGERMARK and M RITZEN The effects of adenosine 5 triphosphate (ATP) on structure and amine content of rat peritoneal mast cells *Exp Cell Res* 1970 *62* 61—75

COFFEY R C Adrenergic control of histamine release from rat mast cells induced by lysosomal cationic protein *Fed Proc* 1970 *29* 742

DAHLQUIST R B DIAMANT and K ELWIN Effect of divalent cations on the interaction of adenosine 5 triphoshate (ATP) with histamine release induced by compound 48/80 *Acta physiol scand* 1973 a *87* 158—167

DAHLQUIST R B DIAMANT and P C KRUGER To be published

DIAMANT B The mechanism of histamine release from rat mast cells induced by adenosine triphosphate *Acta pharmacol* (Kbh) 1967 *25* Suppl 4 33

DIAMANT B and P G KRUGER Histamine release from isolated rat peritoneal mast cells induced by adenosine 5 triphosphate *Acta physiol scand* 1967 *71* 291—302

DIAMANT B and P G KRUGER Structural changes of isolated rat peritoneal mast cells induced by adenosine triphosphate and adenosine diphosphate *J Histochem Cytochem* 1968 *16* 707—716

DIAMANT B and R DAHLQUIST The inhibitory action of adenosine 5 triphosphate (ATP) on histamine release and mast cell degranulation induced by compound 48/80 *Acta pharmacol* (Kbh) 1970 *28* Suppl 1 44

DIAMANT B and C PETERSON The effect of glucose on histamine release from isolated rat peritoneal mast cells induced by adenosine 5 triphosphate *Acta physiol scand* 1970 *80* 299—306

DIAMANT B and C PETERSON The metabolism of monosaccharides in isolated rat mast cells and its influence on histamine release induced by adenosine 5 triphosphate *Acta physiol scand* 1971 *83* 324—334

KRUGER P G B DIAMANT and R DAHLQUIST To be published

MORAN N C B UVNAS and B WESTERHOLM Release of 5 hydroxytryptamine and histamine from rat mast cells *Acta physiol scand* 1962 *56* ?6—41

SAEKI K Effects of compound 48/80 chymotrypsin and antiserum on isolated mast cells under aerobic and anaerobic conditions *Jap J Pharmacol* 1964 *14* 375—390

SUGIYAMA K Calcium-dependent histamine release with degranulation from isolated rat mast cells by adenosine 5 triphosphate *Jap J Pharmacol* 1971 *?1* ?09—?26

SUGIYAMA K Significance of ATP splitting activity of rat peritoneal mast cells in the histamine release induced by exogeneous ATP *Jap J Pharmacol* 1971 a. *?1* ?31—539

SUGIYAMA K and H YAMASAKI Calcium dependent histamine release by ATP from isolated rat mast cells *Jap J Pharmacol* 1969 *19* 1?5—1?6

SHORE P A A BURKHALTER and V H COHN A method for the fluorometric assay of histamine in tissues *J Pharmacol exp Ther* 19?9 *127* 18?—186

THON I L and B UVNAS Mode of storage of histamine in mast cells *Acta physiol scand* 1966 *67* 455—470

UVNAS B and I L THON Isolation of biologically intact mast cells *Exp Cell Res* 19?9 *18* 512—520

UVNAS B and I L THON Evidence for enzymatic histamine release from isolated rat mast cells *Exp Cell Res* 1961 *23* 45—57

Acta physiol scand 1973 87 158—167

From the Department of Pharmacology Karolinska Institutet Stockholm Sweden

Effect of Divalent Cations on the Interaction of Adenosine 5'-Triphosphate (ATP) with Histamine Release Induced by Compound 48/80

By

RUNE DAHLQUIST, BERTIL DIAMANT and KERSTIN ELWIN

Received 21 December 1971

Abstract

DAHLQUIST R B DIAMANT and K ELWIN *Effect of divalent cations on the interaction of adenosine 5 triphosphate (ATP) with histamine release induced by compound 48/80* Acta physiol scand 1973 *87* 158—167

The inhibition exerted by ATP on histamine release caused by compound 48/80 from isolated rat mast cells was counteracted by Ca Mg Ba and Sr but not by Be In addition after the inhibitory effect had been obtained by pretreatment of mast cells with ATP addition of Mg restored the sensitivity of the cells towards 48/80 The spontaneous restoration of the sensitivity of the mast cells towards 48/80 observed after prolonged pretreatment of the cells with ATP (< 10 μM) was enhanced by the presence of glucose Glucose did not effect the inhibition *per se* EDTA blocked 1) the protecting effect of divalent cations on the action of ATP on mast cells in regard to their sensitivity towards 48/80 2) the action of Mg to restore the sensitivity of mast cells towards 48/80 after rendered insensitive by treatment with ATP 3) the spontaneous restoration of the sensitivity of ATP treated mast cells towards 48/80 observed after prolonged incubation alone as well as the enhanced restoration observed in the presence of glucose The results suggest an interaction between divalent cations and ATP on e plasma membrane

Adenosine 5 triphosphate (ATP) exerts various effects on isolated rat mast cells In the presence of Ca^{++} histamine release is induced (Diamant and Kruger 1967, Sugiyama and Yamasaki 1969) In the absence of divalent cations ATP renders mast cells insensitive towards the degranulating and histamine releasing effect of compound 48/80 (Dahlquist *et al* 1973 a) In addition ATP causes changes in the distribution of sodium and potassium over the mast cell membrane effects that are inhibited by increasing concentrations of divalent cations (Dahlquist *et al* To be publ) Furthermore ATP stimulates the uptake of ⁴ Ca to the cells from the incubation medium (Dahlquist and Diamant 1972) Also ATP causes swelling of the mast cells together with certain specific intracellular morphological changes (Diamant and Kruger 1968 Bloom *et al* 1970 Sugiyama 1971) This swelling is counteracted by the presence of Ca 1 mM and above (Kruger *et al* to be publ) In addition

in the presence of 0 25 mM of Ca swelling and intracellular morphological changes of the mast cells precedes a slowly occurring degranulating process which in time is related to the histamine release (Sugiyama 1971 Kruger *et al* to be publ)

The present study was performed in order to further study the characteristics of the inhibition of 48/80 induced histamine release from rat mast cells caused by extra cellularly applied ATP, as well as the mechanism behind the spontaneous cessation of this inhibition observed after prolonged preincubation of the cells with ATP (< 10 mM) (Dahlquist *et al* 1973 a)

A preliminary report on parts of the present study has been presented earlier (Diamant and Dahlquist 1970)

Methods and Materials

Isolation of rat mast cells

Mast cells from the peritoneal and pleural cavities of Sprague Dawley rats (male weight 350—450 g) were isolated by density gradient centrifugation in Ficoll according to the method of Thon and Uvnas (1966) Mast cells collected from the Ficoll were centrifuged ($350 \times g$) and washed three times in room temperature each time with 5 ml of a buffered salt solution containing NaCl 145 mM KCl 2 7 mM and which was buffered to pH 7 0 with 10 % (v/v) Sorensen phosphate buffer ($Na_2HPO_4 + KH_2PO_4$ 67 mM) In addition the buffered salt solution contained human serum albumin 1 mg/ml After washing the mast cells were finally re suspended in 0 3—1 0 ml of the buffered salt solution giving a final concentration of 1 2—3 8 × 10^6 cells/ml The cells were counted in a Burker chamber

Incubation procedure

Isolated rat mast cells were incubated in 2 ml of the buffered salt solution (*v de supra*) at 37 C giving a final concentration varying in different experiments between 2—5 × 10^3 cells/ml.

After preincubation of the cells with ATP for various lengths of time compound 48/80 was added The incubation was continued for an additional 10 min and was terminated by placing the tubes in an ice bath

After incubation the tubes were centrifuged for 10 min at $350 \times g$ at 2—4 C The supernatants were decanted into new tubes and 2 ml of distilled water were added to the cell sediment to extract the remaining histamine

Histamine determination

Histamine was determined by the fluorescence method of Shore *et al* (1959) omitting the extraction steps (Bergendorff 1965)

The fluorescence was measured in a Farrand fluorometer Model A2 or a Farrand ratio fluorometer (Farrand Optical Co Ltd USA)

The histamine release is expressed as percentage appearing in the supernatant of the total histamine content of the cells

The spontaneous release of histamine (*i e* in the absence of compound 48/80 and ATP) amounted to ≤ 7 % and was deducted from all values presented The release obtained by ATP in the absence of 48/80 was subtracted from the histamine release values observed in cell samples exposed to both ATP and 48/80

All samples were run in duplicate

Materials

Compound 48/80 was kindly supplied by AB Leo Helsingborg Sweden and human serum albumin by AB Kabi Stockholm Sweden Ficoll was purchased from AB Pharmacia, Uppsala, Sweden Adenosine 5 triphosphate (ATP) (sodium salt) was obtained from Sigma Chemical Comp St Louis USA Stock solution of ATP (about 0 1 M) was neutralized by the addition of NaOH and assayed spectrophotometrically The stock solution was stored at −20 C The divalent cations investigated were all chloride salts

When necessary the pH of solutions to be used was adjusted to 7 0

TABLE I The protecting effect of Mg^{++} against the inhibitory action caused by ATP on 48/80 induced histamine release Mast cells were preincubated 20 min with 30 or 12 μM of ATP or 2 5 min with 6 μM of ATP in the presence or absence of 1 mM of Mg^{++} prior to the addition of 48/80 (0 6 μg/ml) The histamine release in control samples treated with ATP (30 μM) for 30 min in the absence of 48/80 amounted to between 2–6 % of the total histamine content of the cells

Incubation procedure Mg^{++} (mM)	ATP (μM)	time (min)	48/80 (μg/ml)	time (min)	Histamine release (%) Mean	Range	no of exps
1	30	20	0 6	10	31	28–34	
–	30	20	0 6	10	–1	1–(–2)	2
–	–	20	0 6	10	33	33–34	
1	12	20	0 6	10	32	29–34	
–	12	20	0 6	10	2	1–5	3
–	–	20	0 6	10	31	24–38	
1	6	2 5	0 6	10	37	26–49	
–	6	2 5	0 6	10	4	3–5	3
–	–	2 5	0 6	10	34	27–44	

Results

The protecting effect of divalent cations against the inhibition caused by ATP on histamine release induced by 48/80

When mast cells were exposed to ATP in the presence of Mg^{++} (1 mM), it was found that ATP no longer exerted an inhibitory action of the histamine release induced by 48/80 As evidenced from Table I, this was found to be the case for cells preincubated with 6 μM of ATP for 2 5 min as well as with 12 and 30 μM of ATP for 20 min prior to the addition of 48/80 The protection exerted by Mg was dependent on its concentration in the incubation medium When mast cells were exposed to 12 μM of ATP in the presence of increasing concentrations of Mg^{++} (10^{-5}–6×10^{-4} M) for 20 min prior to the addition of 48/80 (0 6 μg/ml) 0 2 mM of Mg^{++} exerted complete protection of the cells towards the action of ATP as judged from the response of the cells to compound 48/80 (Fig 1) Similarly when mast cells were incubated with 5 μM of ATP together with increasing concentrations of Ca^{++} (6×10 —10^{-3} M) for 2 5 min prior to the addition of 48/80 (0 6 μg/ml) full protection against the action of ATP was noted with 0 4 mM of Ca (Fig 2)

The presence of Ca (1 mM) or Mg (1 mM) offered full protection of the cells against the inhibitory action of ATP on 48/80 induced histamine release and the protecting effect was completely abolished in the presence of EDTA (2 mM) (Table II) Among other divalent cations tested Sr^{++} (1 mM) and Ba (1 mM) incompletely protected the cells against the action of ATP and the effect of these ions was also abolished by the presence of EDTA (2 mM) Be^{++} did not influence the inhibitory effect of ATP on the histamine releasing action of 48/80

In the presence of Ca^{+} (1 mM) histamine release induced by 48/80 (0 3 and 0 6 μg/ml) was significantly enhanced (Table III) This effect was completely counteracted by the presence of EDTA (2 mM) (Table II) Among the other

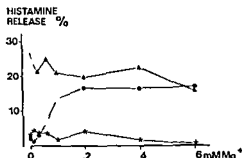


Fig 1

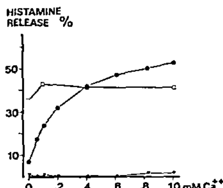


Fig 2

Fig 1 The protecting effect of Mg++ (10⁻⁵—6×10⁻⁴ M) against the inhibition caused by ATP on 48/80 induced histamine release ●——● Mast cells pretreated with ATP (12 μM) in cells treated with ATP (12 μM) in the presence of Mg++ for 30 min. ▲——▲ Mast cells pretreated with Mg++ for 20 min prior to the addition of 48/80 (0 6 μg/ml)

Fig 2 The protecting effect of Ca++ (6×10 —10⁻³ M) against the inhibition caused by ATP on 48/80-induced histamine release ●——● Mast cells pretreated with ATP (5 μM) in the presence of Ca++ for 2 5 min prior to the addition of 48/80 (0 6 μg/ml) ▼——▼ Mast cells treated with ATP (5 μM) in the presence of Ca for 12 5 min □——□ Mast cells pre the presence of Mg++ for 20 min prior to the addition of 48/80 (0 6 μg/ml) ★——★ Mast treated with Ca for 2 5 min prior to the addition of 48/80 (0 6 μg/ml)

divalent cations investigated only Sr seemed to exert a certain degree of enhancement of the histamine release induced by 48/80 and this effect of Sr++ (1 mM) was also counteracted by the presence of EDTA (2 mM) (Table II)

Restoration of the sensitivity of mast cells towards 48/80 by Mg after the cells had been rendered insensitive by treatment with ATP

It was demonstrated that no spontaneous restoration of the sensitivity of mast cells towards the action of 48/80 occurred when dilute cell suspensions had been pre incubated with ATP above 10 μM (Dahlquist *et al* 1973 a) It was therefore of

TABLE II The effect of Be++ Mg++ Ca++ Sr++ and Ba++ (1 mM of each) in the abs nce and in the presence of EDTA (2 mM) on the inh bition caused by ATP (6 μM) of histamine release nduced by 48 80 (0 6 μg/ml) Mast cells were pretreated w th ATP for 2 5 minutes p or to the add tion of 48/80 The divalent cat ons with or without EDTA were present from the beginn ng of the incubation Me n values from two experiments are pre ented

Cat on	H stamine release ( ) No EDTA			EDTA (lmM)		
	48/80	ATP	48/80 after ATP p treatm nt	48/80	ATP	48/80 afte ATP pretr atment
—	29	0	2	31	1	1
Be++	24	3	−1	24	3	0
Mg++	35	0	44	36	1	1
Ca++	59	0	60	34	2	1
Sr +	45	0	25	36	1	−1
Ba++	38	1	4	35	1	1

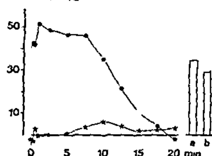


Fig 3

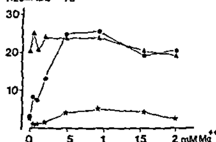


Fig 4

Fig 3 Histamine release induced by 48/80 (0 7 μg/ml) from mast cells pretreated with ATP (12 μM) for various lengths of time prior to the addition of Mg (2 mM) Abscissa Pre incubation time of mast cells with ATP prior to the addition of Mg^{++} After totally 20 min of incubation of mast cells with ATP 48/80 was added (●——●) Zero time denotes the simultaneous exposure of cells to ATP and Mg followed 20 min later by the adddition of 48/80 Time 20 min denotes the simultaneous addition of 48/80 and Mg to cells pretreated with ATP alone for 20 min ★——★ Controls similarly treated with ATP and Mg^{++} but without 48/80 Columns a) and b) denote 48/80 induced histamine release from cells not exposed to ATP in the absence (a) and in the presence (b) of Mg^{++} (2 mM)

Fig 4 Restoration of the sensitivity of mast cells towards 48/80 caused by the addition of increasing concentrations of Mg^{++} (abscissa) after 10 min of preincubation of the cells with ATP All samples were incubated with (●——●) or without (▲——▲) ATP (12 μM) for 10 min prior to the addition of Mg^{++} (5×10^{-5}—2×10^{-3} M) 48/80 (0 7 μg/ml) was added 10 min after the addition of Mg ★——★ Controls similarly incubated but without 48/80

interest to investigate if cells rendered insensitive (by treatment with ATP) towards 48/80 could be made sensitive again by the addition of Mg after the insensitive state had been induced This was indeed found to be the case When Mg^{++} (2 mM) was added to cells after up to 10 min of preincubation with ATP (12 μM) they responded with histamine release when they were subsequently challenged with 48/80 (Fig 3) When mast cells were incubated with 12 μM of ATP for 10 min prior to the addition of Mg (5×10 —2×10^{3} M) and then 10 min later were exposed to 48/80 (Fig 4) it was found that the sensitivity of the cells gradually was restored depending on the concentration of Mg Optimal effect was noted with 0 5—1 mM of the ion

TABLE III The activation induced by 1 mM of Ca^{++} on histamine release caused by 0 3 (7 expts) and 0 6 μg ml (8 expts) of 48/80 Mean and S E are shown The incubation time was 10 min

Histamine release ( )	48/80 0 3 μg/ml (n = 7)	48/80 0 6 μg/ml (n = 8)
No Ca^{++}	25 9±2 3	38 6±2 7
1 mM Ca^{+}	35 4±2 9	61 3±4 0
	P < 0 05	P < 0 001

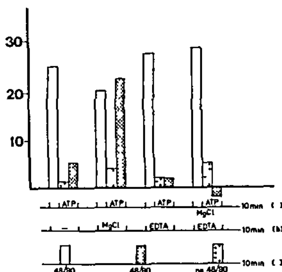


Fig 5 The effect of EDTA on the restoration of the sensitivity of mast cells towards 48/80 induced by Mg on mast cells pretreated with ATP The incubation procedure in this experiment consisted of three 10 min periods (a—c) as presented in the figure

The interaction of EDTA (1 mM) on the restoring effect of Mg^{++} (0 5 mM) on the sensitivity of mast cells towards 48/80 after being rendered insensitive by treatment with ATP (12 μM) for 10 min is shown in Fig 5 As can be noted EDTA neither influenced histamine release induced by 48/80 in samples not exposed to ATP nor influenced the inhibition of 48/80 induced histamine release caused by pretreatment of the cells with ATP When Mg was added to ATP pretreated cells together with EDTA the sensitivity of the cells towards the action of 48/80 was no longer restored by Mg^{++}

The influence of EDTA and glucose on the restoration of the sensitivity of mast cells towards the action of 48/80 observed after prolonged pretreatment with ATP

The inhibitory effect induced by ATP on mast cells in regard to the histamine releasing action of 48/80 was found to disappear spontaneously after prolonged preincubation of the cells with ATU (< 10 μM) (Dahlquist *et al* 1973 a)

The effect of EDTA (1 mM) on the spontaneous restoration of the sensitivity of the mast cells towards the action of 48/80 is shown in Fig 6 Cell samples were preincubated with or without EDTA (1 mM) for 10 min prior to the addition of ATP (5 μM) Following 0 5 2 5 5 10 and 20 min 48/80 (0 6 μg/ml) was added The incubation was terminated 10 min after the addition of 48/80 Time O in Fig 6 denotes the addition of mast cells to a mixture of ATP and 48/80 The results show that EDTA markedly inhibited the spontaneous restoration of the sensitivity of the cells towards the action of 48/80 observed after prolonged incubation of the cells with ATP

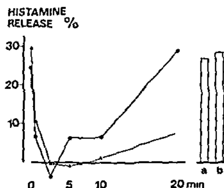


Fig 6 The effect of EDTA on histamine release induced by 48/80 after preincubation of the cells with ATP For incubation procedure is referred to the text ▲——▲ EDTA (1 mM) present Mast cells pretreated with ATP (5 μM) for 0–20 min prior to the addition of 48/80 ●——● Controls similarly incubated without EDTA Columns a) and b) denote histamine release caused by 48/80 from cells not exposed to ATP in the absence (a) and in the presence (b) of EDTA (1 mM) The histamine release in control samples treated with ATP (5 μM) for 30 min in the absence of 48/80 amounted to less than 3 % of the total histamine content of the cells in the absence as well as in the presence of 1 mM of EDTA

In the absence of divalent cations the spontaneous restoration of the sensitivity of ATP treated mast cells towards 48/80 observed after prolonged preincubation with ATP varied markedly in our hands between individual experiments When investigating the possibility that the spontaneous restoration of the sensitivity of the cells towards 48/80 might be influenced by enhancing the cell metabolism it was found that glucose (1 mM) activated the restoration of the sensitivity of the cells towards 48/80 (Fig 7) All cell samples were incubated for 20 min and glucose was present in relevant samples from the beginning of the incubation The cells were exposed to 48/80 (0 6 μg/ml) during the last 10 min of the incubation Therefore samples which were preincubated with ATP (6 μM) for 2 5 min prior to the addition of 48/80 had already been exposed to glucose for 7 5 min before the addition of ATP Glucose was found not to influence the inhibition of 48/80 induced histamine release exerted by pretreatment of the cells with ATP for 2 5 min On the other hand glucose almost completely restored the sensitivity of the cells towards 48/80 after prolonged preincubation (10 min) with ATP in experiments where the spontaneous restoration observed by prolonged preincubation alone (in the absence of glucose) was only small

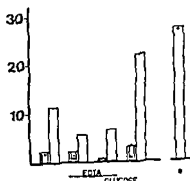


Fig 7 The effect of EDTA and glucose on histamine release induced by 48/80 after pretreatment of mast cells with ATP for 2 5 min (striped bars) or 10 min (open bars) In relevant samples EDTA and glucose were present from the beginning of the incubation The concentrations of the compounds used were 6 μM for ATP 1 mM for glucose 1 mM for EDTA and 0 6 μg/ml for 48/80 Column a) denotes histamine release caused by 48/80 from cells not exposed to ATP The histamine release in control samples treated with ATP (6 μM) together with glucose (1 mM) for 20 min in the absence of 48/80 amounted to 10 % of the total histamine content of the cells In all other instances the histamine release observed in the absence of 48/80 did not exceed i e Mean and individual values of 3 expts

In addition the effect of EDTA (1 mM) is presented regarding histamine release induced by 48/80 after pretreatment of the cells with ATP for 2 5 and 10 min in the absence and presence of glucose (Fig 7) The experiments show that 1 mM of EDTA counteracted the spontaneous restoration of the sensitivity of the mast cells towards 48/80 observed after 10 min of pretreatment of the cells with ATP as well as the enhanced restoration caused by the presence of glucose

Discussion

The present investigation has shown that certain divalent cations (Mg Ca Sr^{++} Ba) completely or partly protected the mast cells towards the inhibitory action of ATP in regard to histamine release exerted by compound 48/80 This could indicate that these ions modify the action of ATP on the plasma membrane An interaction of divalent cations with ATP on the mast cell membrane is also suggested from other experimental data Firstly the swelling of mast cells induced by ATP was counteracted by the presence of 1 mM of Ca (Kruger *et al* to be publ) Secondly the changes in distribution of sodium and potassium over the plasma membrane induced by ATP were counteracted by the presence of 1 mM of Ca or Mg (Dahlquist *et al* to be publ) Further support for an interaction between certain divalent cations and extracellularly applied ATP on the plasma membrane have been presented by Gasic and Stewart (1968) and by Stewart Gasic and Hempling (1969) They found that the volume of TA3 ascites tumour cells increased up to 50 % when exposed to ATP and that the increase in cell volume was prevented by the presence of Ca or Mg^{++} Furthermore cells already swollen by the action of ATP (in the absence of divalent cations) began to shrink back to control size upon the addition of Ca and Mg The effect of ATP was specific and could not be mimicked by other triphosphonucleotides or by EDTA Parallel to the swelling of ascites tumor cells ATP caused a dramatic drop in cell potassium and an even greater rise in cell sodium (Hempling Stewart and Gasic 1969) Thus the findings reported regarding TA3 ascites tumour cells seem to well parallel observations on isolated rat mast cells

Restoration of the sensitivity of the mast cells towards 48/80 was shown to be obtained when Mg was added to cells rendered insensitive by treatment with ATP This indicates that at least after comparative short periods of exposure of the cells to ATP the suggested configurational changes that ATP would induce on the plasma membrane and which resulted in resistance as to the effect of 48/80 could be reversed by Mg This effect of Mg^{++} was completely abolished by the presence of EDTA

The insensitive state induced by ATP towards the action of 48/80 on mast cells was found to spontaneously disappear upon prolonged pretreatment of the cells with ATP ($<$ 10 μM) prior to the addition of 48/80 This effect was not due to ATP decreasing to ineffective concentrations in the medium (Dahlquist *et al* 1973 a)

The spontaneous restoration of the mast cells towards 48/80 observed after prolonged incubation with ATP was found to be facilitated in the presence of glucose On the other hand glucose did not influence the inhibition observed after short periods of ATP pretreatment Both the spontaneous restoration of the sensitivity of the mast cells towards 48/80 observed after prolonged preincubation of the cells with ATP as well as the enhanced restoration caused by glucose was found to be inhibited by EDTA

In summary the present study has revealed the following features in regard to the interaction of ATP with histamine release induced by 48/80

a) Divalent cations like Mg Ca^{++} Sr and Ba but not Be when present together with ATP counteracted the interaction of ATP with the effect of 48/80 EDTA blocked this effect of the divalent cations

b) Mg restored the sensitivity of ATP treated mast cells towards the action of 48/80 after inhibition had occurred EDTA abolished this effect of Mg^{++}

c) Provided the concentration of ATP was less than 10 μM the sensitivity of ATP treated mast cells towards the action of 48/80 was spontaneously restored by prolonged incubation This spontaneous restoration was blocked by EDTA

d) The spontaneous restoration of the sensitivity of ATP treated mast cells towards the action of 48/80 observed after prolonged incubation was enhanced by the presence of glucose This effect of glucose was abolished by EDTA

The experiments might therefore suggest that the spontaneous restoration of the sensitivity of ATP treated mast cells towards the action of 48/80 is an energy dependent process involving a factor which seems to react with EDTA and thus become inactivated The function of this factor might be to counteract configurational changes of the plasma membrane induced by ATP so that it again becomes reactive with 48/80 which would lead to degranulation and histamine release from the cell Such a factor could be a divalent cation present in the mast cell

This study was supported by the Swedish Medical Research Council (grant nr B71 14X 1C04 06A) Karolinska Institutet Stockholm Sweden and Hesselmans Research Foundation Sweden

References

BERGENDORFF A Unpublished observation 1965

BLOOM G D B DIAMANT Ö HAEGERMARK and M RITZEN The effects of adenosine 5 triphosphate (ATP) on structure and amine content of rat peritoneal mast cells *Exp Cell Res* 1970 62 61—75

DAHLQUIST R and B DIAMANT Relation of uptake of sodium and calcium to ATP induced histamine release from rat mast cells *Proc Fifth Int Congress on Pharmacol* (San Francisco) 1972 50

DAHLQUIST R B DIAMANT and K ELWIN Interaction of adenosine 5 triphosphate (ATP) with histamine release induced by compound 48/80 *Acta physiol scand* 1973 *87* 145—157

DAHLQUIST R B DIAMANT and P G KRUGER To be published

DIAMANT B and P G KRUGER Histamine release from isolated rat peritoneal mast cells induced by adenosine 5 triphosphate *Acta physiol scand* 1967 *71* 291—302

DIAMANT B and P G KRUGER Structural changes of isolated rat peritoneal mast cells induced by adenosine triphosphate and adenosine diphosphate *J Histochem Cytochem* 1968 *16* 707—716

DIAMANT B and R. DAHLQUIST The inhibitory action of adenosine 5 triphosphate (ATP) on histamine release and mast cell degranulation induced by compound 48/80 *Acta pharmacol* (Kbh) 1970 28 Suppl 1 44

GASIC G and C STEWART Cell volume regulation in mouse TA3 ascites tumour cells by exogenous ATP as measured by the Coulter Counter *J gen Physiol* [illegible] 242

HEMPLING H G C C STEWART and G GASIC The effect of exogenous ATP on the electrolyte content of TA3 ascites tumor cells *J gen Physiol* 1969 73 133—[illegible]

KRUGER P G B DIAMANT and R. DAHLQUIST To be published

SHORE P A. A BURKHALTER and V H COHN A method for the fluorometric assay of histamine in tissues *J Pharmacol exp Ther* 1959 127 182—186

STEWART C C G GASIC and H G HEMPLING Effect of exogenous ATP on the [illegible] TA3 ascites tumor cells *J gen Physiol* 1969 73 125—132

SUGIYAMA K Calcium dependent histamine release with degranulation from isolated [illegible] cells by adenosine 5 triphosphate *Jap J Pharmacol* 1971 21 [illegible]

SUGIYAMA K and H YAMASAKI Calcium dependent histamine release by ATP from isolated rat mast cells *Jap J Pharmcol* 1969 19 175—176

THON I L and B UVNÄS Mode of storage of histamine in mast cells *Acta physiol scand* 1966 67 455—470

Acta physiol scand 1973 87 168—175

From the Department of Pharmacology Karolinska Institutet Stockholm Sweden

An Attempt to Explain Nervous Transmitter Release as due to Nerve Impulse-Induced Cation Exchange

By

BORJE UVNÄS

Received 23 December 1971

Abstract

UVNÄS B *An attempt to explain nervous transmitter release as due to nerve impulse induced cation exchange* Acta physiol scand 1973 87 168—175

An attempt is made to explain the storage and release of transmitters—particularly in the functional pool—at adrenergic and cholinergic nerve terminals as due to a cation exchange between amines bound to a sulphomucopolysaccharide protein complex in transmitter-containing structures and inorganic ions in the neuro-effector junctional gap It is proposed that the exchange occurs at the nerve terminal membrane vesicle contact area during the period of nerve impulse induced depolarization and increased cation permeability (Fig 7) The theory allows for a total or fractional release of the granule amine stores with or without a concomitant expulsion of the granule matrix Such phenomena as reuptake release by miniature end potentials by amines and by drugs fatigue etc might also be explained on an ion exchange basis

The uptake storage and release of Hi[1] and 5 HT in mast cell granules are processes based on the principle of ion exchange The granules consist mainly of a heparin protein complex with the properties of a weak cation exchange resin with COO groups as the binding sites Accordingly the granules are capable of binding inorganic cations (Na K Ca) as well as biogenic amines (Hi 5 HT A NA DA ACh etc) These inorganic and organic cations compete for common binding sites the resulting occupancy of ionic sites being determined by the affinities of the cations to these sites and their concentrations present The release of Hi and 5 HT from degranulating mast cells can be explained as a stoichiometric exchange at the granule sites between the amines and extracellular cations (Fig 1) (for references see Uvnäs 1964 Uvnäs Åborg and Bergendorff 1970 Bergendorff and Uvnäs 1971 1972 Röhlich Anderson and Uvnäs 1971)

Recent studies in our laboratory have revealed the existence of sulphomucopoly

1 ABBREVIATIONS A Adrenaline NA Noradrenaline CA Catecholamines DA Dopamine ACh Acetylcholine Hi Histamine 5-HT 5-Hydroxytryptamine ATP Adenosine Triphosphate, SMPS Sulphomucopolysaccharide

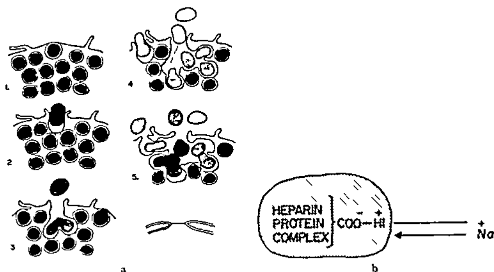


Fig 1 Suggested mechanism of action of compound 48/80 on mast cells a) Exocytosis and partial expulsion of granules (from Röhlich, Anderson and Uvnäs 1971) b) Release of Hi by cation exchange from granules exposed to the extracellular milieu

saccharide (SMPS) protein complexes in all transmitter amine storing vesicle (granule) fractions so far examined, *e g* from adrenals (Fillion, Nosal and Uvnäs 1971), sympathetic nerves, sympathetically innervated organs and brain from rat, cat and dog (Åborg, Fillion, Nosal and Uvnäs 1972). Likewise cholinergic vesicle fractions from the electric organ of *Torpedo marmorata* were found to contain a similar SMPS protein complex (to be published).

The presence in the adrenal chromaffin cells of large amounts of ATP and an acidic protein (chromogranin) and the concomitant release of these components on stimulation of the adrenal medullary secretion by electrical stimuli and by some drugs has led to the assumption that A and NA are stored in an amine ATP protein complex (Carlsson and Hillarp 1956, Blaschko *et al* 1956, Hillarp 1958, Schumann 1958, Stjärne, Hedqvist and Lagercrantz 1970). However, the CA/ATP ratio has been found to exceed 4 thereby excluding an equimolar binding between CA and ATP. Hillarp (1960) and Stjärne (1964) therefore assumed part of the CA to be stored by some different mechanism—in an ATP independent pool. Pletscher and coworkers on the other hand advocate the standpoint—supported by *in vitro* studies—that catecholamines are stored in high molecular ATP/CA aggregates containing also chromogranins and bivalent metals (O'Brien, Da Prada and Pletscher 1972). In such aggregates the CA/ATP ratio is claimed not to be critical for the CA storage function. The opinion that the amine release is the result of an exocytosis (for references see Douglas 1968), an emptying of vesicle contents to the outside of the chromaffin cell, is supported by electron microscopic observations (Diner 1967).

Since adrenergic nerve vesicles also contain large amounts of ATP and a

chromogranin like protein it has been supposed *per analogiam* that the transmitter NA is stored in an ATP protein complex and that it is released via an exocytotic process (for ref see Smith 1971) However also in the nerve granules an ATP independent storage pool has been proposed The two pools behave differently on lysis experiments *in vitro* (Stjarne 1964) on reserpine treatment *in vivo* (Potter and Axelrod 1963) etc

Cholinergic vesicles do not contain appreciable amounts of ATP available for the formation of a transmitter storage complex but their high content of lipids has led to speculations that ACh may be stored in linkage to phospholipids or lipoproteins (Burton 1970)

The now generally accepted view of quantal release of neurotransmitters at cholinergic (Katz 1962), as well as adrenergic (Burnstock and Holman 1961) nerve terminals stems from electrophysiological studies Since the transmitter amines occur packed in vesicles (granules) it has been tempting to identify quantal release with the expulsion of vesicle contents into the neuro effector junctional gap

Some recent reports seem to support the exocytosis concept of transmitter release One of the arguments against this concept has been the apparently too long half life of the transmitter storing granules Their life span was calculated to be 29 days (Dahlstrom 1970) From recent studies on the accumulation of DA β hydroxylase after ligation of splenic nerves in dogs De Potter and Chubb (1971) calculated the vesicular life span to be much shorter between 1 0—1 5 days Such a rapid turn over of the transmitter vesicles is of the same order as that estimated for NA and should then satisfy an exocytotic transmitter release theory Further Weinshilboum *et al* (1971) reported a proportional release of NA and DA β hydroxylase on stimulation of the sympathetic nerves to the vasa deferentia of guinea pigs In addition the ratio of NA and DA β hydroxylase discharged into the incubation medium was similar to that in the soluble portion of the contents of the synaptic vesicles from the vasa deferentia The data were taken by the authors to be compatible with an exocytotic transmitter release process

As to cholinergic transmission Musich and Hubbard (1972) reported a concurrent release of protein and ACh from the curarized mouse diaphragm *in vitro* on stimulation of the phrenic nerve The protein was assumed to originate from cholinergic vesicles of the neuro muscular junctions

However several observations do not accord with a simple exocytotic mechanism A few examples will be given below

Exocytosis at adrenergic or cholinergic nerve terminals has not been convincingly demonstrated by electron microscopy although apposition of vesicles to the juxta junctional terminal membrane has been reported (de Robertis 1964)

It is true that ATP and chromogranin have been detected in perfusates from the spleen after sympathetic nerve stimulation However compared to the NA outflow only minor fractions of the corresponding vesicle contents of ATP and chromogranin were recovered from the perfusates indicating an incomplete emptying of the vesicles involved (Smith *et al* 1970 Stjarne *et al* 1970)

As cited above the formation and transport of amine storing granules to the nerve endings is not considered by Dahlstrom and Haggendal (1970) to be capable of compensating for the granule loss associated with exocytosis

From their own results as well as those of others Folkow *et al* (1967) have calculated the transmitter release per nerve impulse at a vasoconstrictor nerve terminal amount to only about 2×10 of the total NA store Assuming an even distribution of the transmitter release between the varicosities of the terminal the maximal quantal release per varicosity should be around 400 molecules of NA Since it has been calculated that a single adrenergic nerve granule contains about 15 000 NA molecules the release cannot according to Folkow *et al* be explained as a total exocytosis of the content of a single vesicle On the contrary the amount of NA released per impulse should correspond to only a small fraction of the content of a single vesicle Similar arguments were presented in favour of a fractional release of ACh from cholinergic nerve vesicles (Folkow *et al* l c)

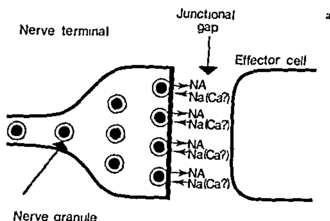
Provided that the calculations above are valid the assumption of a fractional transmitter release seems to be inescapable if a quantal transmitter relea e is responsible also for the miniature end plate potentials in skeletal muscles (Katz 1962) and at sympathetic nerve terminals (Burnstock and Holman 1961)

No adequate hypothesis has yet been advanced to explain such fundamental phenomena in the transmission machinery as the uptake binding and release of the transmitter amine in the assumed ATP protein complex of adrenergic vesicles or in the lipoprotein structures of cholinergic vesicles

The present attempt to explain at least partly transmitter storage and release as the result of cation exchange originates from our observations on the mechanism of the storage and release of Hi 5 HT and other amines by mast cell granules and the discovery of SMPS-complexes in all adrenergic and cholinergic vesicles containing preparations studied (see p 1) These complexes have cation binding properties which are similar to those of the heparin protein complex of the mast cell granules

In electron microscopic studies (Rohlich Anderson and Uvnas 1971) we have observed that the first structural change occurring in a degranulating mast cell is a close apposition of peripherally located granules to the cell membrane A subsequent fusion of the granule membranes and the cell membrane results in the formation of pores which consist of a thin structureless diaphragm bridging the edges of the fused region The entrance of ions (and water?) via these *foci* of increased permeability from the extracellular medium is the probable explanation of the release of Hi from exposed granules and expulsion of part of them (Fig 1)

In the nerve terminals conditions might in some respects be very similar to those in the mast cell if we make the following assumptions The transmitter vesicles contain an SMPS protein complex to which the transmitter amine is — totally or partly — ionically linked As the result of the nerve impulse induced depolarization of the nerve membrane a number of transmitter vesicles become attached to the varicosity membrane The membrane of these vesicles and the varicosity fuse to form transient



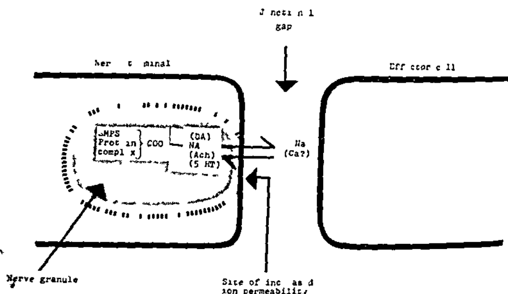


Fig 2 a, b Suggested mechanism of transmitter release from the functional pool at nerve terminal by cation exchange

TABLE I Association Constants (K) and Calculated Maximal Uptake for the Binding of Biogenic Amines Sodium and Calcium in Isolated Granules from Rat Mast Cells (From Bergendorff and Uvnas 1972)

Material	K	Uptake
Calcium	9.1	1010
Histamine	3.8	880
5 Hydroxytryptamine (5 HT)	2.1	840
Tryptamine	1.3	940
Acetylcholine	0.6	1030
Phenylethylamine	0.5	925
Tyramine	0.5	960
Dopamine	0.5	925
Noradrenaline	0.5	925
Sodium	0.5	950
Adrenaline	0.4	950

loci of increased cation permeability Then we have the pre requisite for an influx of cations into the vesicles with an exposure of the SMPS protein complex to these ions

Transient periods of increased cation conductance occur during nerve impulse activity In the frog axon Hodgkin (1963) calculated the sodium ion flux per impulse to 20 000 ions per μ

A transient increase in calcium conductance during the passage of a nerve im pulse over the nerve terminal membrane was demonstrated with a micropipette technique by Katz and Miledi (1967 a b) in their studies on the tetradotoxin treated skeletal muscle and the giant synapse of the squid They suggested that entry of calcium was essential for the transient fusion of the axonal and vesicular mem branes leading to the release of the quantal packets of the transmitter

During the depolarization period a transient general increase in cation permea bility similar to that occurring in the nerve membrane might occur at the sites of membrane fusion

Assuming

a) a varicosity effector cell contact area of 1 μ and a junctional gap width of 1000 Å giving a junctional space with a volume of about 0 1 μ^3

b) a varicosity content of 1500 vesicles with an average diameter of 0 06 μ giving a total vesicle volume of around 0 2 μ^3 (see Folkow *et al* 1967) and

c) affinities of inorganic ions and biogenic amines to the SMPS protein complex of the nerve vesicles of the same order of magnitude as those of the heparin protein complex of mast cell granules (Table I)

then the cations present in the junction gap should after equilibration and even distribution between the vesicles of a varicosity suffice to give intravesicular cation concentrations more than able to release all the amines in the varicosity from an ionic binding to a SMPS protein complex However the transmitter release per impulse has been calculated to amount only to a fraction of the amine content of a varicosity (see above) and release will probably only occur from vesicles in juxta junctional position where the diffusion distance can be neglected Thus there seems to be no difficulty in conceiving a cation exchange with an amine release of the required magnitude to occur from involved vesicles during the depolarization period of a nerve impulse (1 ms) (Fig 2)

According to the version of a cation exchange theory proposed above the size of a quantal package released at a nerve terminal should not depend on the quantity of transmitter in a vesicle but on the ion conductance of the fused vesicle nerve terminal membrane and hence on the characteristics of the ion flux

The reuptake of the transmitter amine could also be explained by such a cation exchange theory it would involve a reversed ion flux probably requiring active transport during the repolarization phase The failing reuptake observed on high frequency stimulation could be due to the fact that there is insufficient time for the required reversed ion transport to take place Explanations based on the above ion

exchange hypothesis might also be found for such phenomena as transmitter release by miniature end potentials fatigue of the release mechanism transmitter release by other amines and by drugs releasable and storage amine pools etc A re evaluation of the functional significance of ATP and chromogranin for the storage and release of amines might be needed However such discussions are better postponed until more qualitative and quantitative information is available about the storage properties of the SMPS protein complexes in the various biogenic amine storing vesicles

The suggestion about the nerve impulse induced transmitter release being based on the simple principle of a cation exchange localized to a storage complex with the properties of a weak cation exchange material is very speculative indeed The suggestion is based mainly on observations made on the properties on *one* biogenic amine store, that in the mast cell However a storage and release mechanism based on ionic exchange is so attractive in its simplicity and applicability that the search for such mechanisms should be rewarding not only for our understanding of the storage and release of biogenic amines but also of other electrically charged products released or secreted from granule stores

This investigation was supported by Swedish Medical Research Council Grant B71 14X 2943 03

References

ABORG C H C FILLION R NOSAL and B UVNAS A sulphomucopolysaccharide protein complex in the adrenergic vesicle (granule) fraction from nerves and tissues *Acta physiol scand* 1972 *86* 427—429

BERGENDORFF A and B UVNAS Storage of 5 hydroxytryptamine in rat mast cells Evidence for an ionic binding to carboxyl groups in a granule heparin protein complex *Acta physiol scand* 1972 *84* 320—331

BERGENDORFF A and B UVNAS Storage properties of rat mast cell granules *in vitro Acta physiol scand* 1973 *87* 213—222

BLASCHKO H G V R BORN A D IORIO and N R EADE Observations on the distribution of catecholamines and adenosin triphosphate in the bovine adrenal medulla *J Physiol* (Lond) 1956 *133* 548—557

BURNSTOCK G and M E HOLMAN The transmission of excitation from autonomic nerve to smooth muscle *J Physiol* (Lond) 1961 *155* 115—133

BURTON R M *Chemical constituents which can bind acetylcholine* Drug and cholinergic mechanisms in the CNS Research Institute of National Defence Stockholm 1970

CARLSSON A and N A HILLARP Release of adenosine triphosphate along with adrenaline and noradrenaline following stimulation of the adrenal medulla *Acta physiol scand* 1956 *37* 235—239

DAHLSTROM A and J HAGGENDAL *Biochemistry of simple neuronal models* New York Raven Press 1970 65—93

DE POTTER W P and I W CHUBB The turnover rae of noradrenergic vesicles *Biochem J* 1971 *125* 375—376

DE ROBERTIS E *Histophysiology of synapses and neurosecretion* Oxford Pergamon Press 1964

DOUGLAS W W Stimulus secretion coupling The concept and clues from chromaffin cells *Brit J Pharmacol* 1968 *34* 451—474

DINER O L expulsion des granules de la medullosurrenale chez le hamster *C R Acad Sci* (Paris) 1967 *D 265* 616—619

FILLION G R NOSAL and B UVNAS The presence of a sulphomucopolysaccharide protein complex in adrenal medullary cell granules *Acta physiol scand* 1971 *83* 286—288

FOLKOW B J HAGGENDAL and B LISANDER Extent of release and elimination of noradrenaline at peripheral adrenergic nerve terminals *Acta physiol scand* 1967 Suppl 307

HILLARP N A Adenosinephosphates and inorganic pho phate in the adrenaline and nor adrenaline cotnaining granules of the adrenal medulla *Acta physiol scad* 1958 *42* 3?1—332
HILLARP N A Different pools of catecholamines stored in the adrenal medulla *Acta physiol scand* 1960 *50* 8—2?
KATZ B The transmission of impulses from nerve to muscle and the subcellular unit to synaptic action *Proc roy Soc B* 196? *155* 455—479
KATZ B and R. MILEDI A study of synaptic transmis ion in the absence of nerve impulses *J Physiol* (Lond) 1967 *192* 407—436
KATZ B and R MILEDI Tetrodotoxin and neuromuscular transmis ion *Proc roy Soc B* 1967 *167* 8—22
HODGKIN A L The coduction of the nervous impulse The Sherrington Lectures VII Liver pool University Press 1963
MUSICH J and J I HUBBARD Biological sciences Release of protein from mouse motor nerve terminals *Nature* 1972 *237* 279—?81
O BRIEN R. A M DA PRADA and A PLETSCHER The ontogenesis of catecholamines and adenosine 5 triphosphate in the adrenal medulla. *Life Sciences* 197? *11* 749—759
POTTER L T and J AXELROD Properties of norepinephrine storage particels of the rat heart *J Pharm exp Ther* 1963 *140* 199—206
ROHLICH P P ANDERSON and B UVNAS Electron microscope observations on compound 48/80 induced degranulation in rat mast cells Evidence for sequential exocytosis of storage granules *J cell Biol* 1971 *51* 465—483
SCHUMANN H J Die Wirkung von Insulin und Reserpin auf den Adrenalin und ATP Gehalt der chromaffinen Granula des Nebennierenmarks *Arch fur Pharmak* 1958 *233* ?37—249
SMITH A D Summing up Some implications of the neuron as a ecreting cell *Phil Trans B* 13 1971 *261* 423—437
SMITH A D W P DE POTTER E J MOLLMAN and A F SCHAEPDRYVER, Release of dopamine β hydroxylase and chromogranin A upon stimulation of the splenic nerve *Tissue and Cell* 1970 2 (4) 547—568
STJARNE L Studies of catecholamine uptake storage and relea e mechanisms *Acta physiol scand* 1964 *62* Suppl 228
STJARNE L P HEDQVIST and H LAGERCRANTZ Catecholamines and adenine nucleotide material in effluent from stimulated adrenal medulla and spleen *Biochem Pharmacol* 1970 *19* 1147—1158
THON I L and B UVNAS Degranulation and histamine release two consecutive teps in the re ponse of rat mast cells to compound 48/80 *Acta physiol scand* 1967 *71* 291—302
UVNAS B C H ABORG and A BERGENDORFF Storage of histamine in mast cells Evidence for an ionic b nding of histamine to protein carboxyls in the granule heparin protein complex *Acta physiol scand* 19 0 Suppl 336
WEINSHILBOUM R M N B THOA D G JOHNSON I J KOPIN and J AXELROD Proportional release of norepinephrine and dopamine β hydroxylase from sympathetic nerves *Science* 1971 *174* 1049—1050

Acta physiol scand 1973 87 176—187

From the Department of Clinical Physiology Karolinska sjukhuset Stockholm Sweden

Circulatory Control via Vagal Afferents

III Control of arterial blood pressure by afferents via the laryngeal communicans with special regard to the restitution after hemorrhage

By

JAN CASTENFORS EVERT KNUTSSON and TORGNY SJÖSTRAND

Received 27 January 1972

Abstract

CASTENFORS J E KNUTSSON and T SJÖSTRAND *Circulatory control via vagal afferents* III *Control of arterial blood pressure by afferents via the laryngeal communicans with special regard to the restitution after hemorrhage* Acta physiol scand 1973 *87* 176—187

The effect of afferent discharges via the laryngeal communicans on the control of the blood pressure has been studied in selected Sprague Dawley rats by cutting the inferior or/and superior laryngeal nerves at varying blood volumes The subsequent changes can be divided into three phases a brief excitatory phase an intermediate and a late phase The intermediate phase is characterized by an increase of heart rate and blood pressure especially marked after hemorrhage The late phase appears after a latency of about 20 min is fully developed in one hour or more and consists at ordinary or decreased blood volume in a lowering and unbalance of the blood pressure If the laryngeal pathway is excluded before or briefly after a blood loss of 30—40 % of the blood volume the pressure is first restored to about 80 % of the initial level but then drops successively until the death of the animal in circulatory insufficiency The effect of laryngeal deafferentation has been compared to the acute effect of hypophysectomia. The similarity of the delayed pressure regression at ordinary and decreased blood volume indicates that the laryngeal nerves act as a pathway for the stimulation of release of ADH vasopressin from the low pressure side

An investigation of afferent discharges via the laryngeal communicans on changes of the blood volume was presented in a previous paper (Castenfors *et al* 1972) It emerged from the study that two different types of impulse discharges can be distinguished one appearing synchronously with the inflow of blood to the heart decreasing in frequency on a successive decrease of the blood volume and disappearing at a given blood loss the other a tonic discharge increasing with hemorrhage Preponderant reasons point to a localisation of the allied receptors to the low pressure side of the circulation We have therefore tentatively suggested that the rhythmically active receptors be called high level receptors of the low pressure side (HL) and the tonically active receptors low level receptors of the low pressure side (LL) The LL receptors have been connected with an increase of the vagal

tone on the heart during hemorrhage demonstrated in the rat, which can be wholly or partly excluded by cutting the laryngeal nerves (Castenfors and Sjostrand 1972) The HL receptors have been assigned a sympathetic influence on the heart and seem to be included in the group of receptors called atrial receptors of type B (Paintal 1953)

The present study was accomplished in order to find out whether the two receptor groups influence the control of arterial blood pressure Owing to the fact that the 2 receptors are predominantly activated at different levels of the central blood volume it was assumed that their effects would be disclosed by cutting the laryngeal path of the vagal afferents at ordinary blood volume and at decreased and increased blood volume The procedure seems to reveal that the LL receptor exerts a significant influence on the blood pressure homeostasis and on its restitution after hemorrhage

Material and Methods

Rats of the Sprague Dawley strain with a b wt. of 250—300 g were used They were anesthetized with pentobarbitone (5—8 mg/100 g b w t) i p In lengthy experiments further anesthetic was given to maintain adequate narcosis

The animals were divided into two groups according to the circulatory reaction on bilateral cutting of the laryngeal nerves and of the vagal trunk respectively The animals of group 1 showed marked changes of the type described in the sequel on exclusion of the laryngeal path way and small changes on bilateral vagotomy Animals of this group have been the main object of this study and unless otherwise stated the observations refer to this group Animals of group 2 showed only moderate changes on laryngeal deafferentation but equivalent changes to those in the first group on bilateral cutting of the vagal trunk Up till now animals of two of about 20 Sprague Dawley breeds have been ascribed to the second group

The procedure and technique employed were the same as described in the previous communications (Castenfors and Sjostrand 1972 Castenfors *et al* 1972) It has been found that in some rats it is necessary to cut both the superior and the inferior laryngeal nerves to get more complete exclusion of the studied afferents The complete exclusion of the laryngeal pathway is in the following called deafferentation

The total blood volume was calculated as 5 5 % of the body weight (Gemzell and Sjostrand 1956) The report is based on more than 40 experiments on an equal number of rats

Results

The effect of exclusion of the laryngeal pathway on heart rate and arterial blood pressure was studied at ordinary blood volume after drawing off usually 25—40 % of the blood volume and after infusion of blood corresponding to 35 % of the ordinary volume or of 3 5 ml isotonic dextrane solution

Deafferentation at ordinary blood volume

A typical example of the effect of cutting the inferior or/and superior laryngeal nerves on heart rate and blood pressure in rats of group 1 is shown in Fig 1 Immediately after the exclusion the heart rate and blood pressure increase After this initial phase lasting a few minutes the blood pressure reaches somewhat higher levels than before the section After a further 15 min the blood pressure decreases successively and 15 min later marked pressure variations appear After about 1 1/2 h a constant mean level is reached The latter period lasts at least for 3 h. The pressure

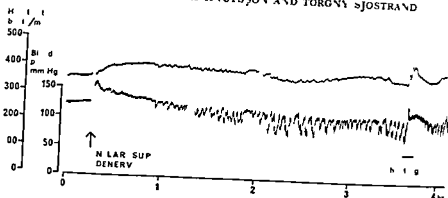


Fig 1 Effect of cutting the superior laryngeal nerve in a rat of group 1 (see text) Increa[se] of heart rate and blood pressure followed by a decrease and irregular waves Stimulation b[y] heating with a lamp provokes a transient increase and stabilization

variations are rather regular giving the appearance of waves of varying wave length

During the late phase the heart rate is somewhat increased and also shows varia tions sometimes synchronously with the variations of the blood pressure After *inter alia* heating the animal the blood pressure may be stabilized for some time as seen in the figure

The effect of deafferentation varies somewhat in different animals of this group but the three phases can generally be distinguished The initial phase seems to cor respond to an excitatory effect of the section and appears in some animals in a decrease of heart rate and blood pressure The intermediate phase can be referred to a change of the vegetative tone on the heart and blood vessels in a sympathetic direction The late effect displays itself predominantly in the pressure curve

Animals of group 2 show small to moderate changes on cutting of the laryngeal nerves but the same changes as described above on bilateral vagotomy caudal to the entrance of the superior laryngeal nerve as appears from Fig 6 and 7

Deafferentation before or after hemorrhage

Animals of group 1

An experiment with cutting the laryngeal pathway just after drawing off 25 % of the blood volume in a Sprague Dawley rat was described earlier (Castenfors and Sjostrand 1972 Fig 7) The heart rate increased transiently after the section and the blood pressure rose from 80 mm Hg to 130 mm Hg The arterial pressure then declined again to 80 mm Hg and showed irregular changes as did the heart rate In three other animals the exclusion of the laryngeal pathway briefly after a blood loss of 25—35 % of calculated blood volume provoked a transient increase of both heart rate and arterial pressure to a level close to the initial followed by a lasting decrease

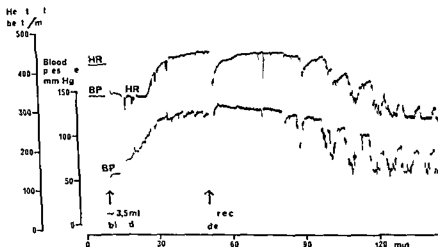


Fig 2 Effect of bleeding a rat 25 % of the calculated blood volume and after 40 min cutting the recurrent nerve Marked fall of heart rate and blood pressure with synchronous waves about 30 min after denervation

Fig 2 shows the effect of cutting the inferior laryngeal nerve 40 min after drawing off 25 % of the blood volume The blood pressure is restored to about 80 % of the level before the section As is seen in the figure the initial effect is a rapid decline of the blood pressure and heart rate The rise of blood pressure in the intermediate phase is less marked than in experiments with deafferentation shortly after hemorrhage However the subsequent decline is greater and a mean pressure level is reached after 1 1/2 h, only somewhat above the level just after the blood loss

In order to find out whether the tolerance to hemorrhage is lowered by exclusion of the laryngeal pathway up to 40 % of the blood volume was drawn before or after deafferentation in a number of animals The upper limit was selected as it was found that rats ordinarily sustained a loss up to this level under similar conditions The experiments are divided into three groups

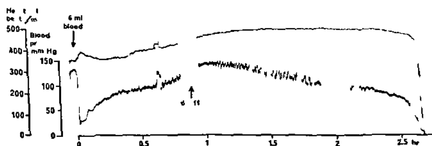


Fig 3 Effect of bleeding a rat of group 1 34 % of the calculated blood volume and after 50 min deafferentation After the initial rise the blood pressure decreases successively and the animal dies about 2 h later
× = occlusion of the carotid arteries

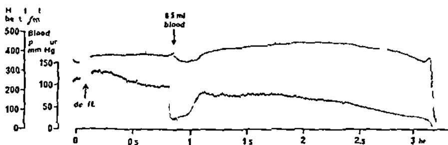


Fig 4 Effect of deafferentation and 45 min later bleeding a rat 36 % of the calculated blood volume The same effect as in Fig 3

1 *Deafferentation after hemorrhage* In three rats 34—40 % of the blood volume was drawn off The deafferentation was performed 40 min to 1 h afterwards when the blood pressure had returned to about 80 % of the initial level The blood pressure then declined gradually and the animals died after 1—3 h as exemplified in Fig 3 A fourth animal was deafferentated two hours after a blood loss of 35 % and in a fifth rat vagotomy was performed between the loss of 34 % and the deafferentation more than 2 h later These rats lived 1 1/2 h afterwards

2 *Initial deafferentation followed by drawing off blood when the new pressure level was reached* The blood pressure was restored to about 80 % of the initial level after a loss of respectively 13 and 26 % of the blood volume in two rats but declined after 30—40 minutes and the animals died one and two hours later respectively see Fig 4

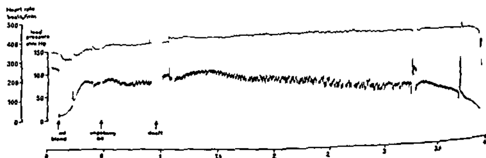


Fig 5 36 % of the blood volume drawn off and 1/2 h later vagotomy bilaterally in a rat of group 1 No effect on the blood pressure A following deafferentation provokes a temporary increase of the blood pressure followed by a successive decrease and [illegible] balance until the death of the animal 3 h later ᴧ = occlusion of the carotid arteries

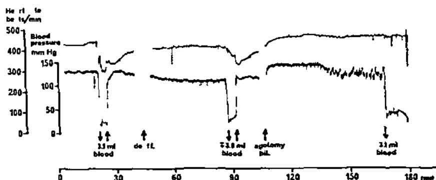


Fig 6 Effect of drawing off and reinfusion of 28 % of the blood volume before and after deafferentation and vagotomy in a rat of group 2 Somewhat smaller decrease of blood pressure and heart rate after deafferentation After vagotomy a temporary increase of blood pressure followed by a decrease with marked waves A rapid drop of the blood pressure after the renewed hemorrhage

3 *Vagotomy caudal to the entrance of the superior laryngeal nerve before or shortly after the blood loss and subsequent deafferentation* In these rats 35 % of the blood volume was drawn off After restitution of the blood pressure to a constant level up to 1 h afterwards the laryngeal pathway was excluded The usual increase of the blood pressure during about 30 min was followed by a decrease and rhythmical pressure variations The animals died 1—3 h later in circulatory insufficiency as exemplified in Fig 5

The experiments indicate that the susceptibility to hemorrhage is increased after cutting the laryngeal nerves in this group of animals as a blood loss up to 40 % of the blood volume is well tolerated by ordinary rats Ten rats were subjected to the same blood loss in experiments with transfusion to another rat or as a consequence of bleeding during the operation None died within 3 h In 2 of the animals the blood pressure was estimated after 3 h or more and was found to be ordinary A definite time interval appeared also between the deafferentation and the successive decline of the arterial pressure

To summarize deafferentation before or less than 2 h after a blood loss of about 35 % of the blood volume provoked in all animals (8) within 3 h a successively developing fatal circulatory insufficiency However deafferentation a longer time after hemorrhage was not fatal which might be explained by a more complete restitution of the blood volume due to influx of water from the interstitial space Thus the hematocrit fell in 2 tested animals from 42 and 44 to respectively 30 and 32 % in 1 h after the blood loss Cutting the vagal trunk below the entrance of the superior laryngeal nerve before deafferentation did not seem to have an obvious effect in this group of animals

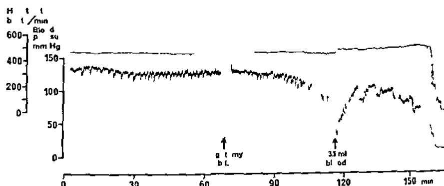


Fig 7 Effect of vagotomy and 40 min later bleeding 28 % of the calculated blod volume in a rat of group 2 After a temporary restitution of the blood pressure a rapid drop until the death of the animal

Animals of group 2

Fig 6 demonstrates the effect of cutting the laryngeal nerves in a rat of group 2 There are no obvious changes of heart rate and blood pressure in the intermediate phase but a small decrease of blood pressure in the late phase A comparison of the effect of blood loss after and before deafferentation discloses a less marked and more slowly appearing decrease of heart rate in direct connection with the blood loss During reinfusion the heart rate decreases which seems to demonstrate a more pronounced influence of the baroreceptors on the high pressure side These changes on blood loss indicate that the LL receptors are only partially excluded

After bilateral vagotomy the heart rate and blood pressure increase markedly in the intermediate phase In the late phase pronounced rhythmical pressure waves appear and the mean pressure level is lowered A blood loss of the same size as before provokes no restitution of the blood pressure but on the contrary a fall after about 10 min Reinfusion of the drawn blood volume causes an increase of the blood pressure far above the initial level (not included in Fig 6) This is later followed by a successive decrease and the death of the animal

Rats belonging to group 2 have sustained a blood loss of about 35 % of the blood volume after exclusion of the laryngeal pathway and have shown a restitution of the blood pressure to or above 80 % of the initial level Vagotomy 1 h or more after the blood loss has provoked a successive decrease and marked variations of the blood pressure 2 animals of 3 have died within 2 1/2 h

If vagotomy has been performed initially a successive circulatory insufficiency has developed—after a roughly 20 min period of compensation—after a blood loss of 28 % of the blood volume in 2 animals The animals have died within 1 h as exemplified in Fig 7

Deafferentation after an expansion of the blood volume

The blood volume has been increased either by blood transfusion or by infusion

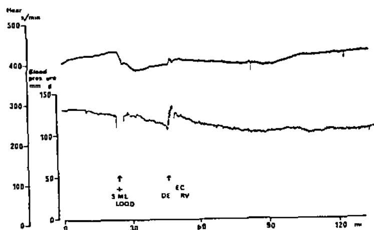


Fig 8 Effect of transfusion of 5 ml blood (33 of calculated blood volume) to a rat of group 1 and 30 min later cutting the recurrent nerve After a transient increase the blood pressure is maintained on a constant level during more than 1 hour

iv or ia of an isotonic dextrane solution in order to increase the blood volume by about 35 % of the original volume With dextrane the blood pressure has under gone a transient marked decrease following the infusion later attaining a somewhat lower level than before During and immediately after the blood transfusion the blood *pressure has shown a small brief increase followed by a period of somewhat* lower pressure level than before the infusion

A typical example of the effect of deafferentation is seen in Fig 8 After the excitatory phase with an increase of heart rate and blood pressure the blood pressure first decreases somewhat During the after phase only small variations of the pressure are seen during 1 1/2 h The heart rate also decreases somewhat during the inter

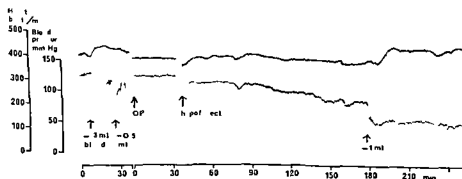


Fig 9 Effect of bleeding a rat before and after hypophysectomy Reinfusion and p eparation for hypophysectomy at the interception of the time scale Su ce s e decrease of the blood pressure about 30 min after hypophysectomy and no restitution after a rather small blood loss.

mediate phase and increases thereafter In 4 other similar expts the heart rate and blood pressure where somewhat lower 1—2 h after the deafferentation

The effect of deafferentation has been the same in 2 expts after dextrane infusion

The effect of hypophysectomy

Fig 9 shows the effect of hypophysectomy on a Sprague Dawley rat After the introduction of the catheters the effect of drawing off 3 1/2 ml blood was tested The usual decrease of the blood pressure was followed by a rapid increase The heart rate was in this case increased on blood loss and decreased after the reinfusion The hypophysis was then laid bare by drilling a hole in the sphenoid and splitting the dura The heart rate and blood pressure were unchanged during the following 30 min Thereafter the hypophysis was removed by suction through a cannula After an immediate drop the blood pressure curve leveled off for about 30 min and thereafter showed a successive decrease to a new level 1 ml of blood was then drawn off and the blood pressure dropped markedly without showing any tendency to restitution during an observation time of more than 1 h.

Some restitution of the blood pressure was observed after hemorrhage in 2 other hypophysectomized animals but the pressure curve levelled off far below the original level The drop in pressure on a given decrease of the blood volume was also more marked than in non hypophysectomized animals

A comparison with the effect of excluding the afferent pathway through the laryngeal communicans discloses a similarity between the 2 conditions in the time course of the pressure drop and the impairment of the restitution after hemorrhage The latter effect seems however to be more marked after hypophysectomy than after exclusion of the laryngeal pathway

Discussion

e laryngeal communicans seems to be a facultative pathway for the afferents from the receptors tentatively located to the low pressure side in the rat To be able to tudy the effects of these receptors on control of the central circulation by deafferentation without obvious interference with other afferents and vagal efferents t is therefore necessary to select suitable animals This has been done in the present study by comparing the effect of exclusion of the laryngeal path in animals from different breeds as animals of the same breed seem to behave in the same manner Vagotomy has been found to provoke similar effects on the blood pressure in the animals which do not react markedly on laryngeal deafferentation It has also been found that in ome animals cutting both of the laryngeal nerves and of the vagus trunk is necessary to induce full effect

The effect on the arterial blood pressure of exclusion of the laryngeal afferents can be divided into 3 phases a brief excitatory phase showing an increase or a decrease of heart rate and blood pressure an intermediate and a late phase both

varying with the blood volume The intermediate phase seems to be mediated by the autonomic nervous system and can be characterized by an increased sympathetic tone especially marked when the section of the pathway is made shortly after hemorrhage After an increase of the blood volume the deafferentation is succeeded by indefinite changes in the intermediate phase

The observations presented in this paper seem to indicate that the LL receptors which are active at ordinary blood volume and are further stimulated by a decrease of the blood volume influence the vegetative tone on the heart and blood vessels thus eliciting a decrease of heart rate and arterial pressure

It has earlier been supposed that depressor fibres from the aorta run in the laryngeal communicans (Andrew 1954 Krieger and Marseillan 1963) The supposition seems to be based on a misunderstanding of neurograms obtained from the laryngeal nerves As pointed out in a previous paper (Castenfors *et al* 1972) the afferent discharges appearing synchronously with the heart rhythm correspond to discharges from atrial receptors of type B (Paintal 1953) which is evident from a comparison with the phonogram It is not possible to establish the mechanical systole from the ECG in the rat as done by Andrew because the end of the T wave does not correspond to the end of the mechanical systole It is therefore necessary to relate the neurogram to the phonogram or to mechanical events The discharges in Andrew s paper appear briefly after the R wave i e during the isometric contraction and the opening of the valves to the pulmonary artery and the aorta and may therefore not be provoked by a stimulation of aortic baroreceptors On an analysis of neurograms from the laryngeal nerves in about 60 rats we have not found discharges which could be allied to arterial baroreceptors In one rat we observed discharges corresponding to atrial receptors of type A It may also be pointed out in this connection that the most marked intermediate effect of cutting the laryngeal nerves appear at a low arterial pressure as after hemorrhage when the arterial baroreceptors are less active or completely inactive The increase of heart rate and arterial pressure on deafferentation may therefore not be due to an exclusion of an arterial depressor tone

The late effect seems to be humorally mediated appears after a latency of about 20 min and develops to its full extent in one hour or more It consists in a lowering and unbalance of the blood pressure at ordinary or decreased blood volume The exclusion of the afferents impairs also the restitution of the blood pressure after hemorrhage and thus seems to increase the susceptibility to blood losses After expansion of the blood volume only small and moderate changes of blood pressure appear during this phase

The most reasonable humoral mediator of the restitution of the blood pressure on hemorrhage is ADH vasopressin which according to several investigations is released on hemorrhage as first observed by Rydin and Verney (1938) The comparison between the effect of deafferentation and hypophysectomia favours this supposition It has been doubted whether ADH has a vasopressin effect at the low concentrations in question but the reported observations are in accordance with

the results of an investigation on the effect of hemorrhage in dogs by Rocha e Silva and Rosenberg (1969). They found *inter alia* on non hypophysectomized dogs an increase of the ADH vasopressin concentration in plasma after hemorrhage to a level which in infusion experiments on hypophysectomized animals gave the same blood pressure increase. Frieden *et al.* (1954) and Weinstein *et al.* (1960) have also suggested an effect of ADH vasopressin on the resistance to hemorrhage.

It has earlier been assumed that the atrial receptors of type B are volume receptors and that they regulate the blood volume via changes of the diuresis mediated by stimulation of the ADH production (Henry and Pearce 1956, Gauer and Henry 1963). However, these receptors are stimulated by an increase of the central blood volume and are not active after a blood loss of some extent. They can only affect ADH production on the condition that there is a tonically active stimulation by other routes and they cannot explain the increase after hemorrhage. The LL receptors seem to fulfil this task. It may be questioned whether the atrial receptors of type B on the whole have any influence on the release of ADH vasopressin as the inhibiting effect of an increase of the central blood volume can be explained by a decrease of the tonic influence of the LL receptors. The assumption that the release of ADH is controlled by the atrial B receptors is based only on the fact that they are located on the low pressure side and that their afferents pass the vagus nerve.

It was earlier (Castenfors *et al.* (1972) suggested that the HL and LL receptors control the blood volume on the low pressure side by affecting the outflow of the blood through influence on the vegetative tone on the heart. The LL receptors seem furthermore, possibly via the hypophysis, to influence the tone of the capacity vessels. The LL receptors seem also to affect the arterial blood pressure directly by influencing the tone of the systemic resistance vessels. By this means they antagonize the effect of the baroreceptors on the high pressure side during restitution after hemorrhage. The significance of this needs additional comments. With a return of the arterial pressure evoked by the baroreceptors the receptor stimulation decreases and thus the elicitation of the nervously mediated mechanism underlying the restitution. Therefore only a partial restitution is possible in this manner. A transfer of the nervously mediated to a hormonally mediated restitution renders further restitution possible. However, this requires that the restitution of the arterial pressure and the distribution of the blood mediated by the vasomotor nerves is moderated. The interaction between the baroreceptors and the LL receptors may facilitate a successive transfer of the initial nervous stimulation predominantely acting on the resistance vessels to an adapted hormonal action on the capacity vessels. This increases the central blood volume to a level so much below the initial as is necessary to maintain an increased hormone release. The nervous control is thereby free for adaptation to other circumstances. This explains *inter alia* the fact that the arterial blood pressure can be normal and sometimes above normal in patients who have lost considerable amounts of blood.

The observations presented in this paper on the function of the receptors presumed to be located on the low pressure side in the control of the arterial blood

pressure are in keeping with the theory of the significance of the central blood volume for the regulation of the circulation and the blood volume (Sjöstrand 1953 1967, Gauer and Henry 1963)

A report of the investigation was given at a joint meeting of the Swedish Association of Physiology and Clinical Physiology on 25th October 1971

References

ANDREW B L A laryngeal pathway for aortic baroreceptor impulses *J Physiol* (Lond) 1954 *125* 352—360

CASTENFORS J and T SJOSTRAND Circulatory control via vagal afferents I Adjustment of heart rate to variations of blood volume in the rat *Acta physiol scand* 1972 *84* 347—354

CASTENFORS J E KNUTSSON and T SJOSTRAND Circulatory control via vagal afferents II Effects of hemorrhage on afferent discharges via the laryngeal communicans in the rat *Acta physiol scand* 1972 *84* 355—365

FRIEDEN J A D KELLER, C P HICKMAN and J H WATERS Decreased resistance to hemorrhage in neurohypophysectomized dogs *Circulat Res* 1954 *2* 214—220

GAUER, O H and J P HENRY Circulatory basis of fluid volume control *Physiol Rev* 1963 *43* 423—481

GEMZELL C A. and T SJOSTRAND The effect of the gonads on the total amount of hemoglobin and blood volume in rats *Acta endocr* (Kbh) 1956 *21* 86—96

HENRY J P and J W PEARCE The possible role of cardiac stretch receptors in the induction of changes in urine flow *J Physiol* (Lond) 1956 *131* 572—585

KRIEGER E M and R F MARSEILLAN Aortic depressor fibres in the rat an electrophysiological study *Amer J Physiol* 1963 *205* 771—774

PAINTAL A S A study of right and left atrial receptors *J Physiol* (Lond) 1953 *120* 596—610

ROCHA E SILVA M JR. and M ROSENBERG The release of vasopressin in response to hemorrhage and its role in the mechanism of blood pressure regulation *J Physiol* (Lond) 1969 *02* 535—557

RYDIN H and E B VERNEY The inhibition of water diuresis by emotional stress and by muscular exercise *Quart J exp Physiol* 1938 *27* 343—353

SJOSTRAND T Volume and distribution of blood and their significance in regulating the circulation *Physiol Rev* 1953 *33* 202—228

SJOSTRAND T *Clinical Physiology* Svenska Bokforlaget Stockholm 1967

WEINSTEIN H R. M BERNE and H SACHS Vasopressin in blood Effect of hemorrhage *Endocrinology* 1960 *66* 712—718

Acta physiol scand 1973 87 188—198
From the Department of Clinical Physiology Karolinska sjukhuset Stockholm Sweden

Circulatory Control via Vagal Afferents

IV Integration of the central control mechanisms in the circulatory adaptation to variations of blood volume

By

JAN CASTENFORS and TORGNY SJOSTRAND

Received 6 March 1972

Abstract

CASTENFORS J and T SJOSTRAND *Circulatory control via vagal afferents* IV Integration of the central control mechanisms in the circulatory adaptation to variations of blood volume Acta physiol scand 1973 *87* 188—198

The interrelations of receptor mechanisms localized to the central part of the cardiovascular system have been studied by alternative exclusion of afferent discharges via the laryngeal pathway vagus trunk and sinus nerve in Sprague Dawley rats An initial increase of arterial pressure followed by a lasting decrease usually coincident with the appearance of pressure waves was found in most animals on laryngeal deafferentation but in others on vagotomy A preceding or subsequent exclusion of afferents via respectively the vagus trunk and laryngeal nerves exerted no or only small effects The pressure response to occlusion of the common carotid arteries was decreased after blood infusions also after hemorrhage to 60 mm *Hg* On a further blood loss to a level of 25 mm *Hg* the response increased Occlusion exclusively of the internal carotids provoked practically the same effect under these conditions On laryngeal deafferentation and vagotomy respectively the pressure response increased Deafferentation after blood infusion especially combined with carotid occlusion and vagotomy provoked forced respiration and in 3 of 6 animals signs of pulmonary edema fatal in two It was concluded that in hypervolemia the circulation is adapted wholly and in hypovolemia in part via afferents running in the laryngeal communicans and/or vagus trunk

Receptors have been localized to the inflow tract of the right and left heart but their functional significance has been obscure (Folkow *et al* 1965) However studies in the rat of afferent discharges via the laryngeal communicans and of the effect of exclusion of this pathway at varying blood volume seem to throw some light on their functional purpose Two kinds of afferent discharges have been described in the laryngeal nerves and are assumed to emanate from receptors on the filling side of the heart one appearing synchronously with the heart rhythm and diminishing with a decrease of the blood volume and another continuous discharge increasing in frequency on hemorrhage (Castenfors *et al* 1972 a) The allied receptors are tentatively called respectively low level (*LL*) and high level (*HL*) receptors of the low pressure side

The LL receptors seem to influence the autonomic nervous tone on the heart in a parasympathetic direction and to have a determinative effect on the tone of the capacity vessels at ordinary or decreased blood volume (Castenfors *et al* 1972 b) Thus they seem to play an important role in the resitution of the blood pressure on a blood loss The function of the HL receptors on the other hand has not been made clear by the e investigations

It seemed worth while to continue the study by alternative exclusion of afferents via the laryngeal communicans vagus trunk and sinus nerve at varying blood volume in order to obtain information about the interrelation of the receptor mechanisms discharging via these pathways The results seem to show that these control mechanisms in some respects act antagonistically but in others in cooperation Furthermore the observations apparently throw some light on the functional purpose of the HL receptors

Material and Methods

Rats of the Sprangue Dawley strain with b w of 250—350 g were used Some experiments were made on animals reared under specific pathogen free conditions (SPF rats) The rats were anesthetized with pentobarbitone (5—8 mg/100 g b w) i p In prolonged experiments further anesthetic was given to maintain adequate narcosis

The procedure and technique employed were the same as described earlier (Castenfors and Sjostrand 1972) In addition occlusion of the internal carot ds was performed by applying a thread around the arteries and exerting a pull To avo d stimulat on of the s nus nerve the thread was drawn out laterally through the skin

Altogether 47 expts on an equal number of animals constitute the basis for the present report

Results

The effect of alternative exclusion of afferents via the laryngeal communicans and the vagus trunk

It has earlier been shown that the effect of exclusion of the laryngeal pathway at ordinary or decreased blood volume varies in Sprague Dawley rats from different breeds and that the effect of vagotomy varies in an opposite direction (Castenfors *et al* 1972 b) This has been confirmed and extended in the present study as demonstrated in the sequel 6 animals of 47 in the present material have been classified as preponderatingly vagal trunk animals In the earlier communication the ordinary animals were denominated as animals of group 1 and the vagal trunk as animals of group 2 The same nomenclature is used in this paper

The general effects in animals of group 1 of cutting the laryngeal pathway at ordinary decreased or increased blood volume are (after an initial excitatory phase) an increase of heart rate and blood pressure (the intermediate phase) followed by a fall in blood pressure simultaneously with the appearance of often very marked pressure waves (the late phase) These effects are especially evident after hemorrhage A subsequent vagotomy has usually only a small effect on the blood pressure

Fig 1 demonstrates the effect of repeated bleeding and reinfusion before and after cutting the laryngeal pathway in an animal of group 1 The same procedure

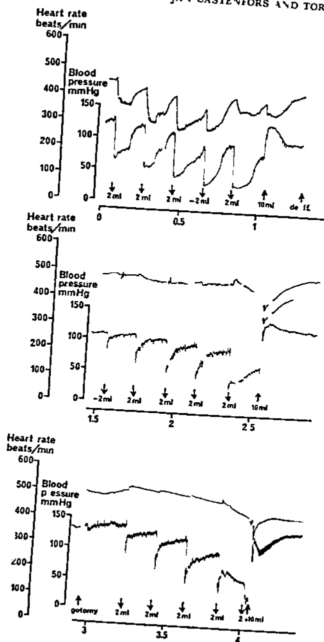


Fig 1 The effect of repeated blood drawings on the heart rate and blood pressure in a Sprague Dawley rat (300 g b w) of group 1 (Top) before (middle) after cutting the laryngeal nerves (bottom) after subsequent vagotomy Blood infusions after each series of blood drawings Continuous time scale in hours For explanation see text

is then repeated after vagotomy Before cutting the laryngeal nerves a decrease in heart rate and a marked depression of the blood pressure with each blood loss appear 5—10 min after the blood drawings the pressure increases coincident with a rise in heart rate After laryngeal deafferentation the heart rate on drawing off the blood does not decrease and the blood pressure is restored rapidly after the blood loss However when more blood is drawn off the restitution appears less effective The picture is similar after vagotomy except for the last two blood losses when the insufficiency of the restitution is obvious

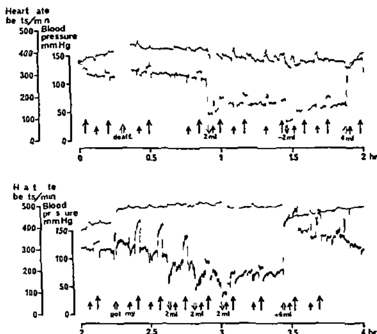


Fig 2 The effect of repeated blood drawings after laryngeal deafferentation (above) and subsequent vagotomy (below) in a rat (297 g b w) of group 2 The small arrows depict occlusion of the internal carotid arteries during one minute the larger arrows occlusion of the common carotid arteries during two minutes Continuous time scale For explanation see text

The conditions are different in Fig 2 which demonstrates the effect of repeated blood loss in an animal of group 2 after deafferentation and vagotomy The restitution of the blood pressure after hemorrhage is slow subsequent to cutting the laryngeal nerves *After vagotomy the fall in blood pressure is much less and the* restitution appears more rapid on comparable blood losses

At increased blood volume the blood pressure first increases somewhat or is unchanged on exclusion of the laryngeal pathway but then decreases to the initial or a lower level (Fig 3 and 4) Vagotomy in animals of group 1 has a transient increasing effect whether it precedes or follows the deafferentation The opposite is the case in animals of group 2 (Fig 5)

Effect of occlusion of the carotid arteries

Intermittent occlusion bilaterally of the common carotid artery has been applied in order to study the effect on the blood pressure of an exclusion of discharges from the carotid sinus baroreceptors The blood pressure response to the occlusion in two rats was compared with the effect of denervation of the carotid sinus It was found that the occlusion caused practically full extinction of the reflex

At ordinary blood volume the effect of occlusion on blood pressure and heart rate

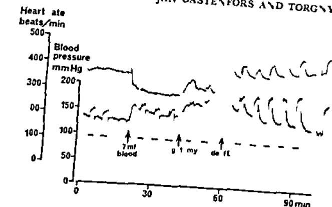


Fig 3 The effect in a group I rat on heart rate and blood pressure of blood infusion corresponding to 36 % of the initial blood volume followed by vagotomy and laryngeal deafferentation The bars depict occlusion of the carotid arteries during two minutes

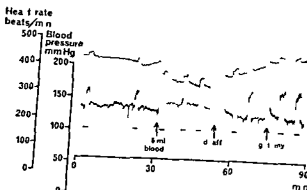


Fig 4 The same as in Fig 3 but deafferentation and vagotomy in changed sequence

varies considerably between animals partly due to variations in the depth of anesthesia

If the carotid arteries are occluded after hemorrhage to a blood pressure between 50 and 30 mm Hg a similar blood pressure response is provoked as at ordinary pressure (Fig 2 and 6) During the subsequent restitution of the blood pressure the response may decrease with increase of the pressure level to about 70 mm Hg On the other hand if blood is successively drawn off the pressure response increases sometimes to a pressure level of about 100 mm Hg

The heart rate response varies usually with the blood pressure response but is sometimes lacking (*cf* Fig 2 and 6)

On repeated hemorrhage the common and the internal carotid arteries have been alternatively occluded It emerges from these experiments that the occlusion of the internal arteries has no definite effect on the blood pressure at ordinary blood volume or on hemorrhage to a blood pressure level of about 80 mm Hg (Fig 7) Between this level and a level of about 25 mm Hg the occlusion of the internal arteries has approximately the same effect as occlusion of the common carotid artery, as is evident from the figure before vagotomy At a lower level occlusion neither of the internal nor of the common artery has any effect Practically the same results have been obtained in three similar experiments The effect on heart

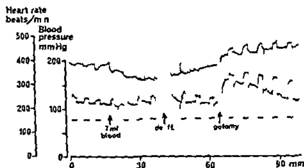


Fig 5 The same as in Fig 4 but in an animal of group 2

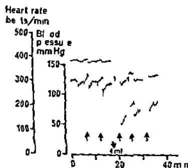


Fig 6

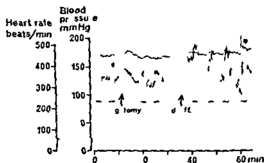


Fig 7

Fig 6 The effect on heart rate and arterial pressure of occlusion of the common carotid arteries during 2 min (arrows) before and after a blood loss corresponding to 30 % of the blood volume

Fig 7 The effect of vagotomy and laryngeal deafferentation on the heart rate and blood pressure response to occlusion of the carotid arteries (bars) in a Sprague Dawley rat of group 1

rate of occlusion of the internal carotids is usually still more marked than on the blood pressure and appears also at higher blood pressure (Fig 2)

These experiments seem to show that the increase of the arterial pressure on occlusion of the common carotid arteries after a blood loss to a pressure level between 60 and 25 mm Hg *i e* at pressures when the baroreceptors are inactive is provoked by the impaired blood flow to the brain

At increased blood volume the pressure response on occlusion of the carotid arteries is decreased (Fig 3 and 4) This is also the case on reinfusion of blood after a blood loss when the blood pressure exceeds the initial level

The heart rate decreases constantly and often to a high degree on blood infusion (Fig 3 4 and 5) On subsequent occlusion of the carotid arteries the heart rate increases but only corresponding to 1/3 to 1/10 of the decrease on infusion as found in experiments on 8 rats

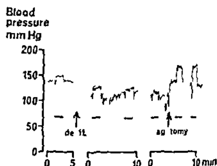


Fig 8 The effect of laryngeal deafferentation and vagotomy on the arterial pressure response to occlusion of the carotid arteries (bars) in a Sprague Dawley rat of group 2

To summarize the pressure response to occlusion of the common carotid arteries decreases with an increase of the blood volume decreases on hemorrhage to a level of 70 mm Hg increases on a further pressure drop down to about 25 mm Hg when it disappears

The effect of occlusion of the carotid arteries before and after cutting the laryngeal nerves and/or vagal trunk

The pressure response to occlusion of the carotid arteries at ordinary blood volume is generally increased after cutting the laryngeal nerves in group 1 animals (Fig 7) The same appears to be the case after vagotomy in group 2 animals (Fig 8) This effect seems to remain for hours As the blood pressure level before occlusion is unchanged or elevated this means that the arterial pressure level after exclusion of the carotid baroreceptors is increased

Thus this level in Fig 7 is 180—190 mm Hg and in Fig 8 about 170 mm Hg during the intermediate phase During the late pha e the mean level like the level after carotid occlusion is decreased

Also after hemorrhage the response after deafferentation is increased even at a low blood pressure (Fig 2) After a subsequent vagotomy the response may be further increased as appears from the same figure before the blood loss

In hypervolemia the pressure response to occlusion of the carotid arteries is usually very marked after laryngeal deafferentation in group 1 animals (Fig 3 and 4) as after vagotomy in animals of group 2 (Fig 5)

The heart rate response to occlusion of the carotid arteries varies after cutting the laryngeal nerves both in hypo and hypervolemia in some animals it is increased in others unchanged The bradycardia on blood infusion usually disappears successively after laryngeal deafferentation (Fig 4)

Vagotomy has no or only a small enhancing effect on the blood pressure response to occlusion of the carotid arteries in group 1 animals This is the case whether vagotomy is performed before or after exclusion of the laryngeal pathway (Fig 3 4 and 7)

After infusion of about 35 % of the calculated blood volume the respiration is often markedly increased on laryngeal deafferentation especially after carotid

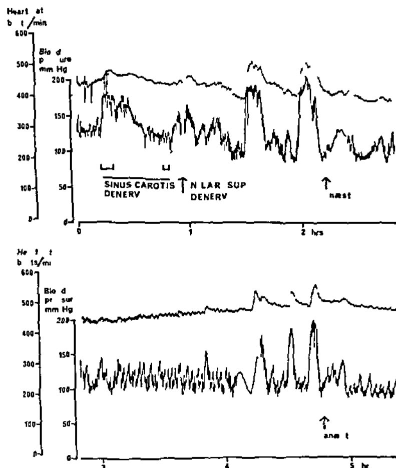


Fig 9 The effect on heart rate and arterial pressure of cutting the superior laryngeal nerves subsequent to denervation of the carotid sinus Large pressure waves of up to 100 mm Hg appear spontaneously seemingly in relation to the depth of narcosis

occlusion This is still more obvious if vagotomy is performed before or after cutting the laryngeal nerves In 3 animals of 6 pulmonary edema appeared and 2 animals died during the attack

It emerges from these observations that after exclusion of the afferents in the laryngeal communicans in animals of group 1 or in the vagal trunk in animals of group 2 the pressure response as well as the level increases on subsequent occlusion of the carotid arteries This effect appears at ordinary decreased or increased blood volume After an increase of the blood volume the cutting of the laryngeal nerves especially in combination with vagotomy and occlusion of the carotid arteries may provoke pulmonary edema The heart rate response to carotid occlusion after de afferentation varies individually but is often increased The bradycardia following

blood infusions is to a great extent abolished by cutting the laryngeal nerves and is only in part influenced by occlusion of the carotid arteries

The effect of exclusion of the laryngeal pathway after denervation of the carotid sinus

Fig 9 shows the effect on the arterial pressure and heart rate of exclusion of the laryngeal pathway in a rat with denervated carotid sinus Cutting of the superior laryngeal nerves is followed by a transient increase of the heart rate and blood pressure Thereafter very marked pressure waves appear some between 90 and 190 mm Hg Anesthesia decreases the pressure waves for some time

Discussion

The observations presented in this paper seem to justify the following conclusions The arterial blood pressure is to an essential degree influenced by discharges in vagal afferents which in the majority of Sprague Dawley rats in our material run in the laryngeal communicans Thus on exclusion of this pathway the arterial pressure increases somewhat after the initial excitatory phase i e during the intermediate phase and then decreases to a maintained level during the late phase The tone exerted by the carotid sinus baroreceptors is simultaneously increased In hypervolemia up to 35 % above the ordinary volume the circulatory adaptation in these animals seems mainly to be accomplished by discharges via the laryngeal afferents Thus the arterial pressure is unchanged or somewhat increased coincident with a depression of the carotid baroreflex However after cutting of the laryngeal pathway the arterial pressure increases markedly on exclusion of the carotid baroreceptor mechanism Under these conditions and especially in combination with vagotomy pulmonary edema may appear On moderate hemorrhage the restitution of the blood pressure is more rapid after laryngeal deafferentation but the tolerance to a larger blood loss is decreased as also shown in an earlier paper (Castenfors et al 1972 b) At ordinary blood volume the blood pressure homeostasis is dependent upon the discharges both via the laryngeal pathway and via the sinus nerve Thus on exclusion of these pathways large pressure waves may appear with an amplitude up to 100 mm Hg In some rats the laryngeal deafferentation has no or only a small influence on the blood pressure control and the above described effects are then provoked by cutting of the vagal trunk

These conclusions are in keeping with the concept of two separate receptor fields controlling the central circulation—the low and high pressure sides (Castenfors and Sjostrand 1972 It is assumed that the receptors on the low pressure side control in the first place the tone of the capacity vessels Thus the LL receptors activated on a decrease of the blood volume seem to increase and the HL receptors activated on a change in the reverse direction to decrease the tone To some extent this effect seems to be mediated by an influence upon the release of ADH vasopressin from the hypophysis However the very rapid adaptation of the capacity vessels on blood infusion suggests a nervous mediation in addition

Besides the action on the capacity vessels the LL receptors exert an influence on the heart in a parasympathetic direction The HL receptors seem to act in the reverse direction by depressing the carotid baroreceptor reflex This conclusion is drawn from the observations that the pressure and heart rate response to occlusion of the carotid arteries usually decrease on blood infusion and increase on subsequent deafferentation However, the decrease of the heart rate on blood infusion call for another explanation Only to a lesser extent is this effect exerted by the carotid sinus reflex. The major part is presumed to be due to the proprioceptive cardiac *reflex elicited by increased filling of the atria and ventricles The laryngeal nerves* may contribute the afferent path of this reflex Thus the effect is abolished to a great extent on exclusion of this pathway In at least one of the neurograms so far recorded from the laryngeal nerves di charges from atrial receptors of type A were recorded (unpublished observations)

During the intermediate phase both LL and HL receptors seem to act antagonistically to the simultaneously activated receptors on the high pressure side Concerning the LL receptors the significance of this antagonism has been discussed earlier (Castenfors *et al* 1972 b) but the significance of the depressive action of the HL receptors needs comment A decrease of the heart rate and thus of the cardiac *output on a stimulation of the baroreceptors subsequent to an increase of the* venous return would further increase the central blood volume and a lowering of the resistance in the systemic circulation might increase the venous return still more and consequently further elevate the pressure in the central veins On the other hand a depression of these effects of the carotid baroreflex would contribute to the restoration of the ordinary volume/pressure relation in the central vessels

The assumed inhibition and stimulation of the release of ADH vasopressin from the hypophysis by respectively the HL and LL receptors would adapt the water diuresis to changes of the central blood volume and total blood volume as suggested by Gauer and Henry (1963)

The reported observations eem also to throw light on the much debated Bainbridge reflex In Sprague Dawley rats as generally in other animals an infusion of blood at ordinary blood volume regularly provokes bradycardia as discu sed above However after hemorrhage the heart rate increases more or less rapidly on reinfusion This is explained by the regre s of the vagal tone evoked by the LL receptors and by the introduction of the depressive effect on the carotid baroreceptors exerted by the HL receptors when they come into play on an increase of the central blood volume Thus *after cutting of the laryngeal pathway the reinfusion consistently* decreases the heart rate This explanation accords with the observations in dog and cat that a prerequisite for obtaining a Brainbridge effect is that the initial heart is depressed *i e* the vagal tone increased (Coleridge and Linden 1955 Jones 1962) However the justification for applying observations from one pecies to another may be called in question but concerning the cardiovascular control the difference between mammals seem to be quantitative rather than qualitative (Korner 1971)

In this connection an investigation by Guazzi *et al* (1962) *should* also be men

tioned From experiments with cutting of vagal nerves in the cat they assumed the existence of a tonic regulation of the arterial pressure via vagal afferents from the cardiopulmonary region

References

CASTENFORS J and T SJOSTRAND Circulatory control via vagal afferents I Adjustment of heart rate to variations of blood volume in the rat *Acta physiol scand* 1972 *84* 34?—354

CASTENFORS J E KNUTSSON and T SJOSTRAND *Circulatory Control via vagal afferents II Effect of hemorrhage on afferent discharges via the laryngeal communicans of the rat Acta physiol scand* 1972 *84* 355—365

CASTENFORS J E KNUTSSON and T SJOSTRAND Circulatory control via vagal afferent III Control of arterial blood pressure by afferents via the laryngeal communicans with special regard to the restitution after hemorrhage *Acta physiol scand 1973 87 1?6—187*

COLERIDGE J C G and R J LINDEN The effect of intravenous infusion upon the heart rate of the anaesthetized dog *J Physiol* (Lond) 1955 *1?8* 310—319

FOLKOW B C HEYMANS and E NEIL Integrated aspects of cardiovascular regulation In *Handbook of Physiology* 1965 section ? vol III 1787

GAUER O H and J P HENRY Circulatory basis of fluid volume control *Physiol Rev* 1963 *43* 4?3—481

GUAZZI M A LIBRETTI and A ZANCHETTI Tonic regulation of the cats blood pressure through vagal afferents from the cardiopulmonary region *Circulat Res* 1962 *11* 7—16

JONES M T The Bambridge reflex *J Physiol* (Lond) 196? 1?0 ?98—305

KORNER P J Integrative neural cardiovascular control *Physiol Rev* 1971 *51* 31?—36?

Acta physiol scand 1973 87 199—207
From the Department of Physiology Veterinarhoegskolan Stockholm Sweden

The Linguo Chorda Tympani Reflex — an Electrophysiologically Undescribed Reflex

By
GORAN HELLEKANT and YASUO KASAHARA[1]

Received 21 April 1972

Abstract

HELLEKANT G and Y KASAHARA *The linguo chorda tympani reflex—an electrophysiologically undescribed reflex* Acta physiol scand 1973 *87* 199—207

The impulse activity in the central part of the chorda tympani nerve of the rat was recorded during mechanical stimulation of the oral region The results obtained at a stimulation rate of 1 Hz show that a) a reflex discharge could be recorded about 6—10 ms after stimulation of the ipsilateral side of the tongue b) stimulation of the contralateral side increased the latency by about 1—1 5 ms c) the reflex could be evoked in animals which showed no spontaneous efferent activity in the chorda tympani nerve d) recordings from nerve filaments showed that a filament could display spontaneous activity without being involved in the reflex e) the lingual nerve serves as the main sensory link on the reflex to mechanical stimulation of the anterior part of the tongue and f) the reflex evoked by stimulation of other parts of the oral region had a longer latency and less intense discharge The results obtained at higher stimulation rate show that a) individual bursts of reflex discharge could be traced at stimulation frequencies up to 60 Hz b) except for a moderate decrease the discharge did not disappear when stimulus frequencies of 5 30 and 50 Hz were used

The chorda tympani nerve is a branch of the facial nerve It joins the lingual nerve during its peripheral course and then supplies the salivary glands and structures in the tongue It consists of an afferent and an efferent part Electrical recordings of its afferent fibres have been obtained in a number of studies since the first one published by Zotterman (1935) Recordings of the efferent fibre activity have recently been published (Hellekant 1971) It has been stated that salivation to a large extent is regulated by the activity in the efferent fibre of the chorda tympani nerve (Emmelin 1967) It is also known that stimulation mechanical or gustatory of the tongue affects the secretion of the salivary glands (*e g* Montgomery 1934) From these observations it can be surmised that the efferent impulse activity of the chorda tympani nerve can be affected by stimulation of the tongue and the oral region This will describe the result of such experiments in the rat

[1] On leave from the Department of Physiology Dental school Osaka Univ Japan

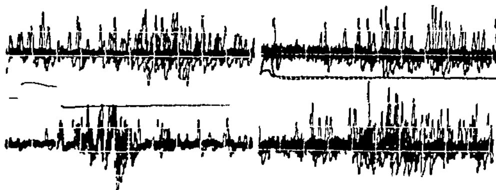


Fig 1 Fig 2

Fig 1 The upper trace shows the efferent impulse activity the lower the afferent response in the cut chorda tympani nerve during a 5 times repeated stimulation of the tongue (middle trace) The record shows that after this stimulation an increase in the efferent chorda tympani activity can be recorded Hypnorm 5 sweeps 2 ms/div

Fig 2 Both records were obtained from the central part of the chorda tympani nerve after stimulation of the same area of the tongue The stimulus used was weaker in the upper trace than in the lower The records show that the stronger stimulus gave the larger discharge Hypnorm 5 sweeps 2 ms/div

Methods

Adult Sprague Dawley rats were used They were anesthetized with Hypnorm® (Leo) Details of the anesthesia are given in a previous study (Hellekant 1971) Gallamine was used to avoid the possible effects of muscular artifacts The animals were then artificially ventilated Some animals were for reasons that will be described later anesthetized with Mebumal® 1 ml of which contains 60 mg pentobarbital sodium One femoral vein was always cannulated and one femoral artery for blood pressure recording The rectal temperature was maintained at 37±1 C The right chorda tympani nerve was dissected and mounted for recording action potentials from the central and sometimes from the peripheral end as well The activity of the nerve was recorded under mineral oil amplified displayed on a Tektronix 5103 double beam storage oscilloscope and then photographically recorded Mechanical stimulation was exerted by a brush of horsehairs connected to a loud speaker that was driven by a stimulator Each stimulus pulse triggered the recording sweep on the oscilloscope The records of this study are composed of 5 to 30 superposed sweeps

Results

General description

The spontaneous efferent chorda tympani activity is characterized by an irregular burstlike discharge of impulse whose amplitude in general is larger than that of the afferent sensory impulses When this efferent activity is recorded during gentle brushing of the surface of the tongue it can be seen to increase Brushing seemed to be a better stimulus than pressing the tongue Stretching gave no effects The increase of efferent activity is more evident if the sweep on the recording oscilloscope is triggered every time the tongue is stimulated the stimulus is repeated and the traces showing the impulse activity caused are superposed Fig 1 is an example of such a record The upper trace shows the activity in the central part of the chorda tympani nerve while the lower one is recorded from the peripheral part The middle trace illustrates the touching of the surface of the tongue with the device described above The stimulus was repeated five times in this case The records were uper

po ed in this as in all records of this study Fig 1 shows that after the stimulation t the tongue surface besides the ensory di charge an increa e in efferent ch rd tympani nerve activity can be recorded It was concluded for several reasons that this increase of efferent activity is a reflex discharge mediated via the CNS Our rea on is the fact that the di charge was recorded from the central end of the cut nerve 6–10 ms after the stimulation had been applied Another is the observation th t cutting the facial nerve between the geniculate ganglion and the brain abolished the e ferent impulses A third reason is that after the ensory nerves from the tongue had been cut the discharge could not be elicited This will be described in more detail later

Relation between the strength of stimulus and the reflex discharge

The efferent discharge increased with increa ed strength of mechanical stimulation Fig 2 shows an example of this. Here the same area of the surface of the tongue was stimulated in each case but the stimulus strength was larger when the lower record was obtained than when the upper one was obtained The stimulation was repeated five times in each record The middle trace illustrates the stimulation Fig 2 shows that the stronger stimulation gave the more intense increase in efferent activity Attempts were not made to quantitate more extensively the relation between stimulus strength and reflex discharge because only one of the many factors which can be expected to affect stimulus strength was controlled i e the movement of the bar of the stimulus device. It is possible that other factors like a new position of the tongue a difference in sensory innervation etc have an influence as soon as the device has been moved

Relation between frequency and duration of stimuli and reflex discharge

The effect of stimulation repeated with shorter inter als than the one usually applied one per sec was also studied Fig 3 shows three recordings during stimulation of the same area of the tongue The same number of stimuli 10 were applied but their rate differed. In the top record the stimulus interval was 1000 ms in the middle one 100 ms and in the bottom 50 ms Fig indicates that a reflex activity was evoked in all three cases and that the number of impulses obtained were about the same

The relation between the reflex activity and the stimulus rate was also studied during extended stimulation The tongue was stimulated for about 15 s with stimulus rates of 5 10 20 50 and 100 Hz The rate applied differed somewhat between the animals. The nerve activity of the central part of the chorda tympani nerve was recorded both directly and via a frequency counter as a histogram Each stimulus frequency was generally applied once The initial maximum frequency of the nerve activity during stimulation was then compared with that after 10 s of stimulation. At all stimulus frequencies a decrease in nerve activity was obtained during the course of stimulation. The decrease was more evident at the higher rates of stimulation but did not exceed 40 %. The possible implications of these results will be discussed later in connection with the question of primary afferent de[illegible]

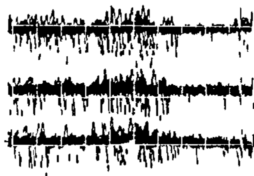


Fig 3

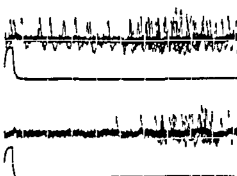


Fig 4

Fig 3 The three records were obtained at stimulation of the same area of the tongue In all three records 10 stimulations were applied but at different rates In the top record the rate was 1 Hz in the middle one 10 Hz and in the bottom one 20 Hz The records indicate little difference in reflex activity between the three stimulation rates used Hypnorm 10 sweeps 2 ms/div

Fig 4 The upper trace was obtained before and the lower after the injection of 0 mg Mebumal/kg b wt The records show that the spontaneous activity disappeared after the barbiturate administration but that a reflex discharge was still elicited though with longer latency and less strength 20 sweeps 2 ms/div

Individual bursts of efferent activity could still be recorded at a stimulation frequency of about 60 Hz When a higher frequency of stimulation was used the individual reflex discharges could not be separated from each other In a similar way the onset and offset of a stimulus gave individual bursts of impulses when the duration was longer than about 30 ms

The relation between place of stimulation and the efferent reflex discharge

It was observed that the latency between stimulus and efferent discharge increased ıen the contralateral side of the tongue was stimulated The difference was about ,1 ms There was also some indication that the reflex discharge was somewhat diminished However because of the possible influence of the factors already touched on this cannot be stated with certainty

The relation between anesthesia and reflex discharge

The ability to record efferent impulses in the chorda tympani nerve was the starting point of this investigation It has been shown (Hellekant 1971) that this activity is dependent upon the anesthesia used No or very few spontaneous impulses can be recorded under Mebumal® anesthesia This was the case in this study too But although no or very few spontaneous impulses were observed a reflex discharge in the central part of the cut chorda tympani nerve could be recorded to touch stimulation of the tongue Fig 4 shows such an example The records of the efferent chorda tympani nerve activity in Fig 4 were obtained before and after the administration of barbiturates The upper trace was obtained under the Hypnorm® anesthesia usually used, while the tongue was stimulated 20 times This trace shows the presence of the spontaneous activity and the reflex discharge caused by the stimulation Then

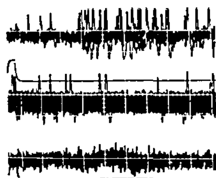


Fig 5



Fig 6

Fig 5 The records were obtained from two different nerve filaments the upper trace from one and the lower two from another The same stimulation was applied during the recording of the upper trace as during the lowest one The middle one shows the spontaneous activity during 0 6 s It can be concluded that no reflex was elicited by the stimulation in the filament that was spontaneously active although judged by the upper trace this stimulation was sufficient to evoke the reflex in another filament Hypnorm 2 ms/div Upper trace 5 sweeps, middle 30 sweeps lower 10 sweeps

Fig 6 The effect of stimulation of the ipsilateral side of the tongue before (upper trace) and after (lower trace) cutting the ipsilateral lingual nerve It can be concluded that the discharge disappeared after the cutting Mebumal 20 sweeps 2 ms/div

20 mg Mebumal®/kg b w t. was injected i v and the same stimulation was repeated The lower trace shows that the reflex could still be elicited though no spontaneous activity was recorded However its latency was increased while its strength was decreased

Observations on efferent chorda tympani nerve filaments

We have not yet made any extensive attempts to discover in which fibre types the reflex described was mediated But we are able to describe a few observations First the results after barbiturate anesthesia show that there is no difficulty in obtaining filaments which show no spontaneous activity but which mediate the reflex Second observations during single fibre dissection indicated that it was easier to obtain a filament that was spontaneously active and mediated the reflex than to obtain a spontaneously active one that did not mediate the reflex However a record of a filament of the last mentioned type is shown in the two lower traces of Fig 5 The tongue was stimulated 10 times in the manner described before while the bottom trace was recorded Fig 5 shows that this stimulation did not elicit any impulses Then the spontaneous impulse activity was recorded during 30 sweeps in the middle trace To test that the mechanical stimulation used was sufficient another filament was put on the electrodes and the same mechanical stimulation was applied 5 times to the tongue while the impulse activity of this filament was recorded in the upper trace From Fig 5 it can be concluded that a fibre can be spontaneously active without mediating the reflex described From this it can be concluded that the reflex described can be elicited in a preparation with no spontaneous efferent impulse activity and that not all spontaneously active fibres were involved in this reflex

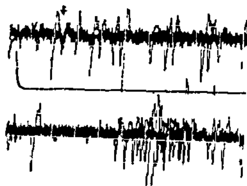


Fig 7

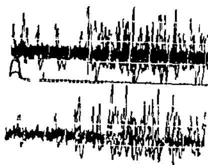


Fig 8

Fig 7 The records were obtained from an uncut chorda tympani nerve in a rat in which the ipsilateral lingual nerve had been cut central to the junctions of the chorda tympani nerve In the upper trace stimulation was applied to the ipsilateral side of the tongue This gave an afferent re ponse (arrow) but no reflex discharge In the lower trace stimulation was applied to the contralateral side which gave a reflex discharge but little or no afferent re ponse in the ipsilateral chorda tympani Hypnorm 5 sweeps 2 ms/div

Fig 8 The reflex discharge elicited by a weaker stimulation of the tongue (upper trace) and the discharge caused by stronger stimulation to the lip (lower trace) The records show a longer latency and less intense discharge to stimulation of the lip than to stimulation of the tongue Hypnorm 5 sweeps 2 ms/div

Observations on the afferent link of the reflex elicited from the tongue

The main ensory nerve of the anterior part of the tongue is the lingual nerve which was left intact during the preceding experiments It is likely that this nerve erves as the main afferent link in the reflex observed This assumption is supported by the results of Fig 6 The efferent chorda tympani nerve activity during mechanical n of one side of the tongue repeated 20 times was recorded before (upper trace) and after (lower trace) the cutting of the ipsilateral lingual nerve Fig 6 shows that cutting the lingual nerve abolished the reflex This indicates that the lingual nerve plays an important role as the sensory link in the reflex studied Fig 7 supports this conclusion and also gives information on the question whether other sensory nerves may serve as afferent links in this reflex Before the results of Fig 7 were obtained the ipsilateral lingual nerve was cut central to the junction with the chorda tympani nerve This left the ipsilateral chorda tympani ner e intact Then the ipsilateral side of the tongue was stimulated mechanically 5 times while the activity in the uncut chorda tympani nerve (upper trace) was recorded The record shows that the stimulation elicited an afferent response in the chorda tympani nerve (arrow) but no definite burst of efferent impulse The other side of the tongue was then stimulated as shown in the lower trace of Fig 7 This gave an efferent discharge The e results suggest that the lingual nerve serves as the main sensory link in the reflex elicited by mechanical stimulation of the anterior part of the tongue

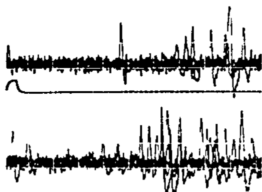


Fig 9 The reflex discharge elicited b stimulation of the chin (upper trace) and close to the lip (lower trace) The records show that the reflex could be evoked from both areas but that its latency was longer and the discharge less intense when evoked by stimulation of the chin Hypnorm 5 sweeps 2 ms/div

Observations obtained during stimulation of other parts of the body

The lingual nerve is a part of the trigeminal nerve A reflex discharge in the efferent chorda tympani nerve might be elicited by stimulation of other areas of the body supplied by the trigeminal nerve This possibility was studied briefly Fig 8 shows the result of such an experiment The efferent chorda tympani nerve activity was recorded during mechanical stimulation repeated 5 times of the tongue (upper trace) and the lip (lower trace) The middle trace illustrates that different strengths of stimulation were used The stronger stimulus was applied to the lip Fig 8 shows that a reflex discharge in the chorda tympani nerve could be elicited by stimulation of another area than the tongue Further it indicates that the discharge caused by stimulation of the lip had a longer latency than that caused by stimulation of the tongue Finally it seems that it was easier to elicit the reflex by tongue stimulation than by lip stimulation because the stronger stimulus gave the smaller effect but the stimulus was applied to two different areas and other factors may have interfered However this conclusion is supported by the records in Fig 9 The efferent chorda tympani activity was recorded during 5 mechanical stimulations of the chin (upper trace) and an area close to the lower lip (lower trace) The records show that a discharge in the efferent chorda tympani nerve could be elicited from both places but that the latency was longer when the reflex was elicited by touching the chin than by touching close to the lip Fig 9 also indicates that the discharge evoked by stimulation of the chin was weaker than that of the lips Other more distant areas like the thoracic and abdominal surface were stimulated but with negative results These results and other indicate that a reflex discharge in chorda tympani activity can be elicited by mechanical stimulation of the area innervated by the trigeminal nerve but that the strength of the reflex elicited decreased with the distance from the tongue

In summary the results show that a reflex discharge in the efferent chorda tympani nerve fibres can be elicited by mechanical stimulation of the oral region The strongest reflex with the shortest latency was obtained after stimulation of the ipsilateral side of the tongue The reflex can be elicited in animals which show no

spontaneous efferent chorda tympani nerve activity Not all efferent fibres in the chorda tympani nerve take part in this reflex

Discussion

It is known that stimulation of sensory nerves to the trigeminal nuclei can give rise to antidromic impulses in other sensory nerves (*cf* Dubner and Kawamura 1971) These antidromic impulses are caused by a depolarization of pre synaptic sensory nerve endings (*cf* Eccles 1964) The phenomena is called primary afferent depolarization (PAD) It could be suggested that the reflex activity described in this study was the result of PAD However some observations are not in agreement with such an assumption The PAD caused by a single sensory stimulation reaches its maximum 50 to 100 ms after the stimulation and lasts for about 200 ms (Stewart Scibetta and King 1967) During this period the ability of the sensory nerve to initiate and propagate impulses is diminished or abolished This means that if the activity described was the result of antidromic impulses this activity would fade away when the stimulus was applied at a sufficient high frequency The result described and shown in the Fig 3 did not indicate this Therefore, we think that there is little reason to regard the reflex described as the result of antidromic impulses in sensory nerves

About 40 % of the fibres in the chorda tympani nerve are efferent (Foley 1945) They supply salivary glands and probably also structures in the tongue Of the salivary glands the submandibular and sublingual ones are to a large extent controlled by this nerve (Emmelin 1964) However an intact efferent nerve supply to these glands is not the only condition for their secretion The sensory nerves from the oral cavity to the CNS must also be intact (Emmelin 1967) Thus after sensory [illegible]ervation by cutting of the lingual nerve salivation diminished (Emmelin 1961) [illegible] intact animal mechanical stimulation of the oral region elicits salivation (*e g* Montgomery 1934) while salivation more or less stops during sleep (Schneyer *et al* 1966) when no or few oral sensations are evoked It has been suggested by earlier studies (*cf* Emmelin 1964) that the salivary glands receive a permanent inflow of secretory impulses via the chorda tympani nerve and that these impulses are elicited from the salivary nuclei as a result of sensations from the oral region mediated by the lingual nerve These observations together with those of the present study lead us to suggest that the reflex described which we call the linguo chorda tympani reflex occurred in salivary fibres and that it is part of the basis for a normal flow of saliva We are not prepared to suggest that this reflex forms the whole basis for a normal flow of saliva because pilot experiments show that a decrease of the efferent chorda tympani activity can be recorded together with an increased salivation from the submandibular gland This problem will be the topic for a later study Further if this hypothesis is correct then this type of stimulation may be used to evoke central salivary neuronal activity which apparently has not yet been achieved (Wang 1964)

Fig 5 shows that the impul e activity of some of the efferent chorda tympani nerve fibres was not affected by mechanical stimulation of the tongue It can of course be suggested that stimulation of another area of the oral region would have elicited a reflex in these fibres However it is unlikely that mechanical stimulation of other parts of the oral region would have been more effective since stimulation of the tongue seemed to be the most effective stimulus (Miller 1913) If this is correct there are fibres in the efferent part of the chorda tympani nerve whose impul e pattern is not affected by the same kind of stimulus This may be regarded as an indication that there are fibres with different functions The possibility of vasodilator fibres in the chorda tympani nerve is of interest in this context Fitzgerald and Alexander (1969) describe in the tongue of the cat parasympathetic ganglia whose preganglionic fibres came from the chorda lingual nerve and whose postganglionic fibres could be traced to small arteries No relation to salivary structure was observed This has been discus ed previously (Hellekant 1972) In conclusion this discussion suggests that the reflex described is part of the basis for the flow of saliva that is constantly secreted in the waking state

This study was supported by a grant from Svenska Maltdrycksforskningsinstitutet and by the Swedi h Medical Research Council grant No B72 14X 2467 05 A Y Kasahara was supported in part by the Naito Research Grant of Japan for 1971

References

DUBNER R and Y KAWAMURA *Oral facial sensory and motor m chanisms* Appleton Century Crofts 1971 pp 1—384

ECCLES J C *The physiology of synapses* Springer 1964 pp 1—316

EMMELIN N Sensitization of the submaxillary and parot d glands by removal of part of the afferent inflo v to the salivary nuclei *J Physiol* (Lond) 1961 *157* 402—409

EMMELIN N Secretory nerves of the sal vary glands In *Salivary glands and their secretions* 1964 p 161—173

EMMELIN N Nervous control of sali ary glands *Handbook of Physiol* Sec 6 1967 2 595—632

FITZGERALD M J T and R W ALEXANDER The intramuscular ganglia of the cat s tongue *J Anat* 1969 *105* 27—46

FOLEY J O Tl e sensory and motor axons of the chorda tympani *Proc Soc exp Biol* (N Y) 1945 *60* 262—267

HELLEKANT G Efferent mpul es in the chorda tympani nerve of the rat *Acta physiol sca d* 1971 *83* 203—209

HELLEKANT G Circulat on of tl e tongue In *O al Physiol gy* Pergamon Press 1972 p 12 —137

MILLER F R On the reaction of the sali ary centres *Q art J exp Physiol* 1913 *6* 57—72

MONTGOMERY M F Studie upon secretory activity of gland of the oral and pharyngeal mucous membranes *Proc Soc e p Biol* (N Y) 1934 *31* 717—719

NAKAMURA Y P imary aff r nt depolarizat on n the lingual nerve as evoked by high threshold afferents from the masseter m uscle of the cat In *Oral fa al sen ory and moto mechanisms* Appleton—Century—Crofts 1971 p 251—261

SCHNEYER, L H W PIGMA L HANAGAN and R W GILMORE Rate of flo of human parotid sublingual and bn a illar sec etions during sleep *J dent Res* 1956 *35* 109—114

STEWART D H JR C J SCIBETT and R B KING Presynapt c inhibition in the trigeminal relay nucle *J Neurophy i l* 1967 *30* 135—153

WANG S C Central n rvou rep entation of salivary ecretion *Saliva y glands and the r se ret s* Pergamon P ess 1964 p 145—159

ZOTTERMAN Y Action potentials in the glossopharyngeal nerve and in the chorda tympani *Ska d A ch Phy ol* 1935 72 73—77

Acta physiol scand 1973 87 208—212

From the Department of Medical Chemistry University of Turku Finland

Metabolism of Fracture Callus of Rat in vitro

II Incorporation of ^{35}S-Sulphate, ^{3}H-Proline and ^{32}P-Phosphate

By

R Penttinen

Received 5 May 1972

Abstract

Penttinen R *Metabolism of fracture callus in vitro II Incorporation of ^{35}S sulphate ^{3}H proline and ^{32}P phosphate* Acta physiol scand 1973 *87* 208—212

The incorporation rate of ^{35}S sulphate into the glycosaminoglycans of callus *in vitro* was at the highest on the 7th day after the fracture corresponding with the proliferation of cartilage Protein synthesis per callus occurred at the maximal rate 4 days later (during the transition of cartilaginous to ossifying callus) as judged from the radioactivities of ^{3}H hydroxyproline and ^{3}H proline in collagen and other proteins

Radioactive phosphate accumulated maximally into the cartilaginous matrix on the 7th day and into the osseous matrix on the 21st day after the fracture No differences in activities were detected between heated and intact slices indicating non enzymatic uptake of phosphate ions from the medium

Studies on bone healing *in vivo* with ^{35}S sulphate, ^{45}Ca ^{32}P phosphate or ^{3}H *proline* (McLean and Urist 1961 Udupa and Singh 1966 Lemaire 1966 Shtacher and Firschein 1967 Singh *et al* 1968) have demonstrated that the isotopes are rapidly incorporated into glycosaminoglycans minerals and proteins of the growing callus Both regenerating and original bone were usually included in the analyzed samples While the metabolism of bone and cartilage has been studied *in vitro* (Krane *et al* 1967 Nichols *et al* 1969) no data are available on callus which is the object of this study with special emphasis on its various ages

Material and Methods

Animals and materials The preparation of rat tibial calluses for the incubations is described earlier (Penttinen *et al* 1972 a Penttinen 1972) Slices from each callus were incubated in one closed 50 ml centrifuge tube at 37 C for 6 h in a metabolic incubator (A Gallenkamp and Co Ltd London England) One callus from each series of 8 tibias was heated on a boiling water bath for 15 min before the incubation to get a metabolically inactive control The radioactive isotopes were purchased from the Radiochemical Centre Amersham Bucks England The radioactivities were counted in a Packard Tri Carb Model 3370 liquid scintillation spectrometer and corrected for decay

Incorporation of ^{35}S sulphate The slices were incubated in 3 0 ml *Krebs Ringer phosphate* medium pH 7 4 with 22 4 mM glucose and 0 1 *mg/ml ampicillin* (Doctacillin Astra Swedeal

in air atmosphere Magnesium chloride (1 1 mM) was used instead of magnesium sulphate (Boström et al 1963) After the preincubation of the samples for 30 min 10 μCi of carrier free $Na_2{}^{35}SO_4$ (SJS 1) was injected in 100 μl 0 9 % NaCl solution After 6 h sodium monoiodoacetate (I Light & Co Ltd England) was added in 100 μl of water to a final concentration of 5 10⁻³ M The mixture was cooled homogenized in 13 0 ml of absolute ethanol with an Ultra Turrax homogenizer (TP 18/2 Germany) for 3×5 s centrifuged at 20 000×g and at 4 C for 30 min and the precipitate washed twice with 5 ml of 80 % (v/v) aqueous ethanol The sediment was extracted twice with 10 ml of chloroform methanol (2 1 v/v) and air-dried The residues were then digested at 65 C with 5 mg of papain (60 000 units/g No 7144 water soluble E Merck A G Germany) in 5 0 ml of 0 1 M sodium acetate buffer pH 5 5 containing 0 05 M Na_2EDTA and 0 005 M cysteine hydrochloride After further 10 h incubation the samples were sedimented at 20 000×g for 30 min, washed once with 1 0 ml of the buffer and the supernatants combined The radioactivities in aliquots of 100 or 200 μl were counted in 100-fold volume of the solvent which consisted of 6 vol of freshly distilled ethylene glycol monomethylether (methyl cellosolve Shell) and 10 vol of scintillation fluid (15 g 1 5-diphenyloxazole and 50 mg of 4 bis-2 (5 phenyloxazolyl) benzene, Packard Instrument Co U S A in 1000 ml of distilled toluene)

The glycosaminoglycans of the combined supernatants were precipitated with 4 vol of absolute ethanol containing 2 % (w/v) potassium acetate left for 16 h at 4 C and centrifuged at 35 000×g for 30 min The sediments were resuspended in water and after repeated precipitation dissolved in 2 5 ml of water and counted for the activity of ³⁵S sulphate as before

The contents of uronic acids of the samples were estimated as described before (Penttinen et al 1972 a) Sulphate was determined according to Antonopoulos (1962) The coefficients of variation of the uronic acid and sulphate assays were ±5 2 % and ±4 5 % respectively

Incorporation of ³H proline After preincubation of the slices for 15 min in 5 0 ml pH 7 4 Krebs Ringer phosphate buffer with 22 4 mM glucose and 0 1 mg/ml ampicillin 80 μCi of universally labelled ³H L-proline (TRA 82) 100 mCi/mM in 100 μl of 0 9 % NaCl solution was added to the incubation mixture After 6 h the samples were cooled and trichloroacetic acid was added to 5 % (w/v) The samples were homogenized with 10 mg of inactive L-proline (puriss Fluka AG Buchs Switzerland) with the Ultra Turrax homogenizer for 5×5 s and dialyzed for 72 h against 1000 fold volume of ice-cold distilled water with daily changes The material was centrifuged at 35 000×g and 4 C for 30 min The radioactivity of hydroxyproline of the sediment was determined as described by Juva and Prockop (1966) The counting efficiencies for tritium were calculated by using ³H hexadecane as standards quenched with increasing amounts of acetone The quenching of each sample was corrected by external standardization The coefficient of variation of the method was ±2 6 %

Incorporation of ³² P phosphate To obtain a high specific P activity in the phospholipids a low phosphate Krebs-Ringer bicarbonate medium with 22 4 mM glucose and 0 1 mg/ml of ampicillin was used It was gassed with 95 % O_2+5 % CO_2 for 15 min in 50 ml centrifuge tubes prior to the use After a preincubation of the slices for 15 min in 3 0 ml of the buffer 100 μCi of sodium ³²P-orthophosphate (PBS 1) was added in 100 μl of 0 9 % NaCl The incubation was stopped after 6 h by adding 12 1 ml of chloroform methanol (1 2 v/v) and homogenizing the samples with the Ultra Turrax homogenizer for 3×5 s After standing for 15 min 4 0 ml of chloroform was added and the homogenization was repeated Then 4 0 ml distilled water was added and the two layers settled The chloroform layer was removed the slices were washed twice with 2 0 ml of chloroform filtered and dried at 37 C

The lipid free sediments were then washed twice with cold water centrifuged at 20 000×g dialyzed in separate vials for 72 h against 1000 fold volumes of distilled water at 4 C and centrifuged at 35 000×g at 4 C The sediments were suspended in 10 ml of 6 N HCl and hydrolyzed at 130 C for 3 h The hydrochloric acid was removed by evaporation and the residue dissolved in 10 ml of distilled water The radioactivities of the samples and standards were counted in 20 ml of the methyl cellosolve scintillation fluid mixture. The analyses on phospholipids will be published separately

Results

The amount of glycosaminoglycan bound sulphate per callus increased during days 3—14 after the fracture (Fig 1) Highest radioactivities of incorporated ³⁵S sulphate in the glycosaminoglycans were found on the 7th day of healing (Fig 2) which

Acta physiol scand 1973 87 208—212

From the Department of Medical Chemistry University of Turku Finland

Metabolism of Fracture Callus of Rat in vitro
II Incorporation of ^{35}S-Sulphate, ^{3}H-Proline and ^{32}P Phosphate

By

R Penttinen

Received 5 May 1972

Abstract

Penttinen R. *Metabolism of fracture callus in vitro II Incorporation of ^{35}S sulphate ^{3}H proline and ^{32}P phosphate* Acta physiol scand 1973 *87* 208—212

The incorporation rate of ^{35}S sulphate into the glycosaminoglycans of callus *in vitro* was at the highest on the 7th day after the fracture corresponding with the proliferation of cartilage Protein synthesis per callus occurred at the maximal rate 4 days later (during the transition of cartilaginous to ossifying callus) as judged from the radioactivities of ^{3}H hydroxyproline and ^{3}H proline in collagen and other proteins

Radioactive phosphate accumulated maximally into the cartilaginous matrix on the 7th day and into the osseous matrix on the 21st day after the fracture No differences in a tissues were detected between heated and intact slices indicating non enzymatic uptake of phosphate ions from the medium

Studies on bone healing *in vivo* with ^{35}S sulphate ^{45}Ca ^{32}P phosphate or ^{3}H proline (McLean and Urist 1961 Udupa and Singh 1966 Lemaire 1966 Shtacher and Firschein 1967 Singh *et al* 1968) have demonstrated that the isotopes are rapidly incorporated into glycosaminoglycans minerals and proteins of the growing callus Both regenerating and original bone were usually included in the analyzed samples While the metabolism of bone and cartilage has been studied *in vitro* (Krane *et al* 1967 Nichols *et al* 1969) no data are available on callus which is the object of this study with special emphasis on its various ages

Material and Methods

Animals and materials The preparation of rat tibial calluses for the incubations is described earlier (Penttinen *et al* 1972 a Penttinen 1972) Slices from each callus were incubated in one closed 50-ml centrifuge tube at 37 C for 6 h in a metabolic incubator (A Gallenkamp and Co Ltd London England) One callus from each series of 8 tibias was heated on a boiling water bath for 15 min before the incubation to get a metabolically inactive control The radioactive isotopes were purchased from the Radiochemical Centre Amersham Bucks England The radioactivities were counted in a Packard Tri Carb Model 3320 liquid scintillation spectrometer and corrected for decay

Incorporation of ^{35}S sulphate The slices were incubated in 3 0 ml Krebs-Ringer phosphate medium pH 7 4 with 22 4 mM glucose and 0 1 mg/ml ampicillin (Doctacillin Astra Sweden)

After an incubation of the callus slices with ³ P phosphate high activities were detected in the lipid free samples on the 7th 14th and 21st days of healing The samples of the 9th and 11th days were less labelled (Fig 4) The differences be tween the values of the 5th and 7th or 7th and 9th days were statistically highly significant ($p < 0\,001$ t test) The activities of the heated samples were equally high The activities in phospholipids in the heated slices were very low and are not presented in details

Discussion

The synthesis of glycosaminoglycans and proteins

The ratios of sulphate proline or sulphate hydroxyproline activities were maximal on the 7th day of healing indicating that the synthesis of glycosaminoglycans preceded that of collagen and other proteins This agrees with data on the accumula tion of sulphate and proline into callus *in vivo* (McLean and Urist 1961 Udupa and Singh 1966 Shtacher and Firschein 1967) The highest ratios of hydroxyproline to the total activity and RNA ribose to DNA (Penttinen *et al* 1972 b) were detected on the 11th day after the fracture indicating maximal synthesis of collagen and other proteins The consumption of oxygen and production of lactate by the slices of the single callus were maximal on the 7th—9th day of healing (Penttinen 1972) indicat ing higher energy metabolism during the most active glycosaminoglycan synthesis than during the maximal synthesis of proteins Collagen accounted for 6—11 % of the total protein synthesis as judged from the amounts of incorporated proline and hydroxyproline This finding agrees with the results on mammalian fibroblasts (Green *et al* 1966 Goldberg and Green 1968) or experimental granulomas (Lampiaho and Kulonen 1967) The incorporation experiments carried out *in vitro* on calvariae of osteopetrotic mice (Marks 1969) or on embryonic chick tibiae (Juva 1968) resulted in figures of 20—25 % As about 60—90 % (dry weight) of the organic matrix of cartilage and bone is collagen these findings indicate its slow turnover in callus The radioactivities of proline and hydroxyproline correlated significantly to each other ($r = 0\,824$ $p < 0\,001$ t test) due to the simultaneous synthesis of collagen and other proteins but showed no correlation to the amounts of total nitrogen or hydroxyproline The correlations between the radioactivities and amounts of sulphate were statistically insignificant in a similar way

Accumulation of ³ P phosphate into the matrix

The peak in the radioactivity of ^{32}P phosphate on the 7th day coincided with the highest activity of ^{35}S sulphate This shows the active interaction of the newly synthesized cartilaginous matrix and phosphate minerals at that day The non lipid and phospholipid radioactivities in heated and intact slices indicate that non lipid phosphate accumulated into the matrix by adsorption precipitation or ex change with the medium and independently of living cells

I am grateful to Professor E Kulonen M D for his advice This study has been supported by a personal grant from the Emil Aaltonen Foundation and institutional grants from the Sigrid Jusélius Foundation

References

ANTONOPOULOS C A A modification for the determination of sulphate in mucopolysaccharides by the benzidine method *Acta chem scand* 1962 *16* 1521

BOSTROM H A MORETTI and M WHITEHOUSE Studies on the biochemistry of heart valves I On the biosynthesis of mucopolysaccharides in bovine heart valves *Biochim biophys Acta* (Amst) 1963 *74* 213—221

GOLDBERG B and H GREEN Relation between collagen synthesis and collagen proline hydroxylase activity in mammalian cells *Nature* (Lond) 1969 *221* 267—268

GREEN H G J TODARO and B GOLDBERG Collagen synthesis in fibroblasts transformed by oncogenic viruses *Nature* (Lond) 1966 *209* 916—917

JUVA K Hydroxylation of proline in the biosynthesis of collagen An experimental study with chick embryo and granulation tissue of rat *Acta physiol scand* 1968 *Suppl 308*

JUVA K and D J PROCKOP Modified procedure for the assay of ^{3}H or ^{14}C labelled hydroxyproline *Analyt Biochem* 1966 *15* 77—83

KRANE S M V PARSONS and A S KUNIN Studies of the metabolism of epiphyseal cartilage In *Cartilage Degradation and Repair* Proceedings of a Workshop held on 3—5 Nov 1966 pp 43—58 Ed C A L Bassett Natl Acad Sci and Natl Res Council Washington 1967

LAMPIAHO K and E KULONEN Metabolic phases during the development of granulation tissue *Biochem J* 1967 *105* 333—341

LEMAIRE R G Calcium metabolism in fracture healing An experimental kinetic study in rats using Ca^{45} *J Bone Jt Surg* 1966 *48 A* 1156—1170

MARKS S C Jr The specificity of the ^{3}H proline incorporation test as a measure of bone matrix formation *Biochem biophys Res Commun* 1969 *35* 236—242

MCLEAN F C and M R URIST *Bone An introduction to the physiology of skeletal tissue* 2d edition 1961 The Univ of Chicago Press Chicago

NICHOLS G JR B FLANAGAN and J VAN DER SLUYS VEER Distortions of bone cell metabolism in uremia and their cause *Arch intern Med* 1969 *124* 530—538

PENTTINEN R Metabolism of fracture callus of rat in vitro I Oxygen consumption and lactic acid production *Acta physiol scand* 1973 *87* 133—137

PENTTINEN R, J NIINIKOSKI and E KULONEN Hyperbaric oxygenation and fracture healing A biochemical study with rats *Acta chir scand* 1972 a *138* 39—44

PENTTINEN R J NIINIKOSKI J RANTANEN and E KULONEN Effects of hyperbaric oxygenation and reduced barometric pressure on the nucleic acid contents of healing fractures A biochemical study with rats *Acta chir scand* 1972 b *138* 269—274

SHTACHER G and H E FIRSCHEIN Collagen and mineral kinetics in bone after fracture *Amer J Physiol* 1967 *213* 863—866

SINGH L M R J DELLA ROSA and J E DUNPHY Mobilization of calcium in fractured bones in rats *Surg Gynec Obstet* 1968 *126* 243—248

UDUPA K N and R H SINGH Isotopic studies on the effect of certain anabolic and anti anabolic hormones on the turnover of minerals and mucopolysaccharides in healing of fractures *Indian J med Res* 1966 *54* 932—941

Acta physiol scand 1973 87 213—222

From the Department of Pharmacology Karolinska Institutet Stockholm Sweden

Storage Properties of Rat Mast Cell Granules In vitro

By

ANDERS BERGENDORFF and BORJE UVNÄS

Received 9 May 1972

Abstract

BERGENDORFF, A and B UVNÄS *Storage properties of rat mast cell granules in vitro* Acta physiol scand 1973 *87* 213—222

Basophil granules were isolated from rat peritoneal and thoracic mast cells The granules were depleted of their endogenous Hi and 5-HT The uptake of Hi 5-HT TrpA PhEA TA DA NA ACh Na and Ca (for abbreviations see page 215) by the depleted granules the release of Hi from Hi recharged granules by Ca Na and K and the competition between NA and Hi ACh and NA ACh and Hi for the granule storage sites were studied The results support the hypothesis that these granule stores have the properties of a weak cation exchange resin Furthermore it was found that amphetamine and ephedrine competed with NA for the storage sites The results indicate an unspecific granule storage of cations—organic and inorganic—differing only in their affinities for the binding sites

The cation storing capacity of histamine (Hi) and 5 hydroxytryptamine (5 HT) containing granules of rat peritoneal mast cells can be ascribed to their main constituent a heparin protein complex (Bergqvist Samuelsson and Uvnas 1971) The complex has the properties of a weak cation exchange material with COO groups as binding sites In uptake studies *in vitro* sodium (Na) Hi and 5 HT were observed to compete for these sites (Uvnas Åborg and Bergendorff 1970 Bergendorff and Uvnas 1972)

In the present *in vitro* studies we have investigated the ability of isolated mast cell granules to store various biogenic and non biogenic amines as well as inorganic cations As to be expected from the unspecificity of the granule storage mechanism indicated by our previous observations cited above all the organic and inorganic cations studied were taken up by the granules the ions apparently competing for common binding sites

Methods

Isolation of mast cells

Mast cells were isolated from the abdominal and thoracic cavities of male Sprague Dawley rats weighing 350—450 g as described by Thon and Uvnas (1967) However after gradient centrifugation in Ficoll (30 and 40) the cells were not washed in sucrose solution but in isotonic salt solution containing 1 mg human serum albumin per ml.

Preparation of amine depleted granules

Granules were isolated from rat mast cells by differential centrifugation after lysis of the cells in deionized water The granules were depl ted of Hi and 5 HT by suspension in 10 mM NaCl solution The Na taken up in exchange for the amine was then removed by repeated washing of the granules in deionized water according to the procedures described by Uvnas *et al* (1970)

Uptake of Hi 5 HT TrpA PhEA TA DA NA A ACh Na and Ca
(for abbreviations see page 215)

Depleted and dried granules from 120 rats (about 25 mg) were suspended in deionized water and divided into 12 aliquots Each aliquot was again dried over P_2O and weighed Each lot of granules (about 2 mg) was resuspended in deionized water (0 1—0 13 mg/100 μl) and 100 μl aliquots of the suspension were pipetted into tared polypropylene tubes To these tubes were added amine sodium or calcium solutions to the desired concentrations and the corresponding labelled material (^{14}C labelled amine ^{22}NaCl or $^{45}CaCl_2$) An uptake curve usually comprised the concentration range bet een 0 1—30 mM In each experiment a Na or Hi uptake curve was used as a reference

After incubation for 15 min at room temperature the samples were centrifuged at 3000 x *g* for 20 min the supernatants carefully sucked off as completely as possible and their pHs measured The tube with its precipitate was weighed to allow calculation of the amount of contaminating radioactivity in the remaining incubation solution The granule precipitate was dissolved in 0 5 ml of 1 M NaOH and the radioactivity recorded in an aliquot by liquid scintillation spectrometry For further details see Uvnas *et al* (1970)

Hi
10 mM Hi dihydrochloride was titrated to pH 7 with 10 mM Hi base ^{14}C Hi was added to each incubation sample to give a final radioactivity level of 0 22 μCi per ml

5-HT
To 133 mg of 5HT hydrogen oxalate was added 35 mg of $Ca(OH)_2$ in 25 ml of deionized water The precipitate was rapidly removed by centrifugation The supernatant was immediately adjusted to pH 7 with 0 2 N H_2SO_3 and diluted with deionized water to a 10 mM 5-HT stock solution ^{14}C-5 HT was added to all incubation samples to give a final radioactivity level of 0 22 μCi per ml

PhEA and TrpA
These two compounds neither of which are hydroxylated were dissolved in a small volume of deionized water (PhEA hydrochloride 414 4 mg TrpA hydrochloride 138 13 mg) put on a Dowex 2 X8 column and eluted with 20 ml of deionized water The eluate was immediately adjusted to pH 7 with 0 2 N H_2SO_3 and diluted to 50 ml giving a 50 mM solution of PhEA and a 10 mM solution of TrpA ^{14}C PhEA or ^{14}C-TrpA was added to each incubation sample to give a final radioactivity level of 0 22 μCi per ml

TA NA and A
The free bases of TA (207 mg) DL-NA (253 5 mg) and DL-A (274 5 mg) in 30 ml of deionized water were immediately titrated to pH 7 with 0 2 N H_2SO_4 and diluted to 50 ml giving 30 mM solutions in each case To each incubation sample was added ^{14}C-TA or ^{14}C DL A to give a final radioactivity level of 0 22 μCi per ml or ^{14}C DL-NA to give a final radioactivity level of 0 11 μCi per ml

DA and ACh
DA hydrochloride and ACh chloride were dissolved in deionized water to give 30 mM solutions To each incubation sample was added ^{14}C labeled compound to give a final radioactivity level of 0 22 μCi per ml (^{14}C-ACh) or 0 11 μCi per ml (^{14}C-DA)

Calcium
$CaCl_2$ was dissolved in deionized water giving a solution containing 30 meq Ca/L $^{45}CaCl_2$ was added to all incubation samples to give a radioactivity level of 0 51 μCi/ml

Sodium
30 mM sodium dih drogenpho phate (= 30 meq Na/L) was titrated with 15 mM disodium hydrogenphosphate (= 30 meq Na/L) to pH 7 ^{22}NaCl was added to each incubation sample to give a final radioactivity level of 0 2 μCi per ml

Competition for binding sites

1 Release studies
"Depleted" granules were recharged with Hi by incubation in 0 1 mM Hi solution (pH 7) containing ^{14}C-Hi the chosen concentration giving about 30 % saturation of the binding sites

TABLE I Association constants (K_{ass}) and maximal uptake values (U_{max}) for biogenic amines and the inorganic cations Na and Ca calculated from their semilog uptake curves (For details of the calculations see Bergendorff and Uvnäs 1972)

	K_{ass} (1/mM)	U_m (mμmoles/mg
Histamine	3.8	880
5 Hydroxytryptamine	2.1	840
Tryptamine	1.3	940
Acetylcholine	0.6	1030
Phenylethylamine	0.5	925
Tyramine	0.5	960
Dopamine	0.5	925
Noradrenaline	*0.5*	*925*
Adrenaline	0.4	950
Sodium	0.5	950
Calcium	9.1 (1/meq/L)	1010 (mμeq/mg)

(Uvnäs *et al* 1970) After incubation the samples were centrifuged at $3000 \times g$ for 20 min and their supernatants sucked off The tubes with their precipitates and remaining incubation medium were weighed in order to allow correction for contaminating radioactivity The Hi charged granules were suspended in NaCl, KCl or $CaCl_2$ solutions (0.01—30 meq/L) for 15 min and centrifuged at $3000 \times g$ for 20 min The supernatants were sucked off and the radioactivity in aliquots recorded The precipitates were dissolved in 0.5 ml of 1 M NaOH and the radioactivity recorded In another series of experiments the granules were recharged in 0.3 mM Hi solution (pH 7) containing ^{14}C-Hi This concentration gave a Hi saturation of about 50 % These Hi containing granules were then incubated in 0.1—10 mM 5 HT solutions and the Hi released measured

Materials Histamine (ring 2 ^{14}C) dihydrochloride Sp act 51 mCi/mmol Tyramine 1^{2} ^{14}C hydrochloride Sp act 44 mCi/mmol Dopamine-^{14}C hydrochloride Sp act 55 mCi/mmol DL-Noradrenaline (carbinol ^{14}C) DL-bitartrate Sp act 28 mCi/mmol DL-Adrenaline (carbinol ^{1} C) DL-bitartrate Sp act 27 mCi/mmol Acetylcholine (methyl ^{14}C) chloride Sp act 54 mCi/mmol ^{22}NaCl Sp act 1 mCi/mg $^{45}CaCl_2$ Sp act. 4 mCi/mg The Radiochemical Centre Amersham England 5 Hydroxytryptamine-2 ^{14}C binoxalate Sp act 17 mCi/mmol Tryptamine 2 ^{14}C bisuccinate Sp act 8.9 mCi/mmol β Phenylethylamine-1 ^{14}C hydrochloride Sp act. 3.6 mCi/mmol New England Nuclear Boston Mass USA Ficoll AB *Pharmacia* Uppsala Sweden Human serum albumin (free from preservati es) AB Kabi Strangn s Sweden All other substances were obtained from usual commercial sources

Abbreviations Hi Histamine 5 HT 5 Hydroxytryptamine TrpA Tryptamine PhEA Phenyl ethylamine TA Tyramine DA Dopamine NA Noradrenaline A Adrenaline ACh Acetyl choline Eph Ephedrine Amph Amphetamine

B Studies on competitive uptake

The mutual influence of various amines on each othe s uptake was studied by the simultaneous exposure of depleted granules to two amines Uptake curves for the one amine (with the admixture of ^{14}C-labelled amine) were determined in the absence and in the presence of the other amine in concentrations found to exert a significant uptake inhibition.

Results

Uptake of amines

All the biogenic amines tested (Table I) were taken up by the granules *in vitro* in other words not only the endogenous mast cell amines Hi and 5 HT but also the transmitter amines ACh A NA DA and the related amines PhEA and TA. All the uptake curves showed very similar courses They usually leveled off at extra

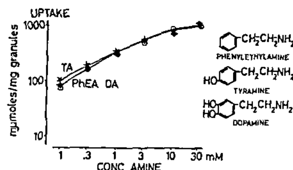


Fig 1 Uptake by mast cell granules of PhEA (O—O) TA (★—★) and DA (■—■) Note that hydroxylation of the aromatic ring has no effect on the affinity for the storage sites

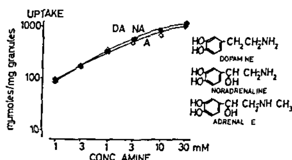


Fig 2 Uptake by mast cell granules of DA (■—■) NA (■—■) and A (◇—◇) Note that hydroxylation and methylation of the side chain have no significant effect on the affinity for the storage sites

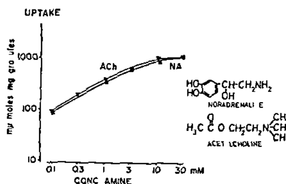


Fig 3 Uptake by mast cell granules of NA (■—■) and ACh (▼—▼) Note the similarity in affinity for storage sites between the primary amine (NA) and the quarternary amine (ACh)

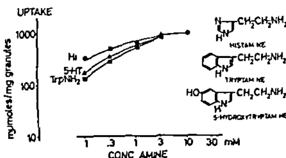


Fig 4 Uptake by mast cell granules of Hi (●—●) 5-HT (▲—▲) and TrpA (□—□)

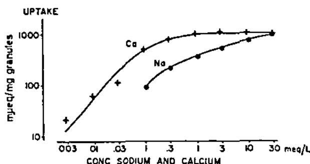


Fig 5 Uptake by mast cell granules of Na (o—o) and Ca (+—+) Note that Ca[2] have a higher affinity than Na ions for storage sites

granular amine concentrations around 10—30 mM Within this concentration range the uptake approached 1000 mμmoles/mg (Fig 1 2 3, and 4)

For unknown reasons some granule preparations showed a lower uptake capacity although only exceptionally below 750 mμmoles/mg In order to get more comparable storage characteristics for the various amines such low uptake curves were corrected From the concomitant Na or Hi reference curve (see p 2) the amine uptake values were recalculated to correspond to a Na or Hi uptake of 1000 mμmoles/mg at 30 mM concentration After such recalculation it is evident (Fig 1 2 3 and 4) that the uptake curves for the phenylethylamine derivates (PhEA TA DA NA A) show almost identical courses Accordingly the calculated association constants (K) and the maximal uptake values (U_m) were found to be around 0 5 and 925—950 mμmoles/mg respectively for this family of amines

The degree of hydroxylation of the aromatic ring (PhEA<TA<DA Fig 1) or of the ethylamide side chain (DA<NA Fig 2) did not noticeably change the K of the amines for the granule binding sites nor did the transformation of the primary amino group to a secondary one by methylation (NA to A Fig 2)

In spite of its completely different structure ACh shows the same uptake characteristics as the phenylethylamine derivatives (Fig 3)

The amines which contain an indol or imidazole ring (TrpA 5 HT and Hi) formed a group with somewhat different uptake characteristics The affinities for these amines (K_{as} TrpA 1 3 5 HT 2 1 Hi 3 8) were higher than those of the amines already discussed The uptake of the amines reached 1000 mμmoles/mg within the same amine conc range (10—30 mM) as the catecholamines (Fig 4)

Uptake of Na and Ca

Of the 2 cations studied Na ions had an affinity for the granule storage sites similar to that of catecholamines (K 0 5) while Ca ions showed a considerably higher affinity (K 9 1) the highest K found in this study However the maximal uptake for the 2 ions was the same 1000 mμeq/mg The U_m for Ca ions was reached already at 1 meq/L which reflects their high K while the corresponding saturation for Na ions was reached at 10—20 meq/L (Fig 5)

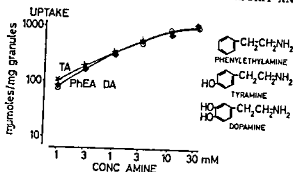


Fig 1 Uptake by mast cell granules of PhEA (○—○) TA (★—★) and DA (■—■) Note that hydroxylation of the aromatic ring has no effect on the affinity for the storage sites

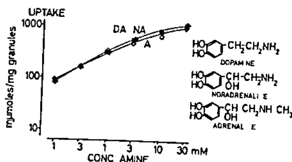


Fig 2 Uptake by mast cell granules of DA (■—■) NA (■—■) and A (◇—◇) Note that hydroxylation and methylation of the side chain have no significant effect on the affinity for the storage sites

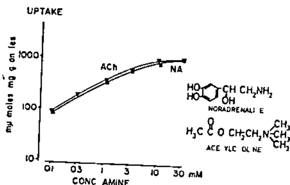


Fig 3 Uptake by mast cell granules of NA (■—□) and ACh (▼—▼) Note the similarity in affinity for storage sites between the primary amine (NA) and the quarternary amine (ACh)

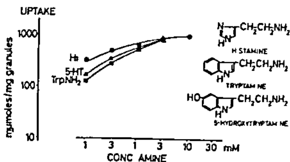


Fig 4 Uptake by mast cell granules of Hi (●—●) 5 HT (▲—▲) and TrpA (□—□)

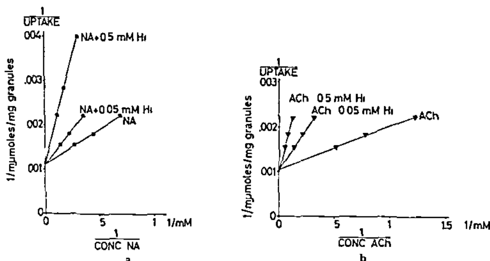


Fig 8 Lineweaver Burk plots illustrating the competition for granule storage sites between endogenous biogenic amines a) NA (■—■) and Hi b) ACh (▼—▼) and Hi c) (▼—▼) and NA

Note the results show competition for common binding sites between structurally unrelated amines

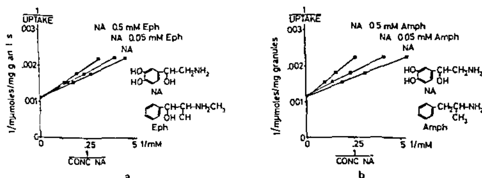


Fig 9 Lineweaver Burk plots illustrating the competition for granule storage sites between endogenous and exogenous amines a) NA (■—■) and Eph b) NA (■—■) and Amph

occurred Hi inhibited the uptake of NA (Fig 8 a) and of ACh (Fig 8 b) and the uptake of ACh was inhibited by NA (Fig 8 c)

Exogenous amines also competitively inhibited the uptake of endogenous biogenic amines the uptake of NA was inhibited in the presence of ephedrine (Fig 9 a) or amphetamine (Fig 9 b)

Discussion

We have earlier presented evidence (Uvnas *et al* 1970 Bergendorff and Uvnas 1972) to show that the endogenous mast cell amines Hi and 5 HT are stored ionically bound to the complex between heparin and a basic polypeptide which forms the matrix of the mast cell granules The granule matrix has the properties of a weak cation exchange material Quantitative studies on the uptake of Hi, 5 HT and Na ions as well as titration for acid groups in the granules, led to the conclusion that the amines (and the Na ions) are stored linked to COO groups, mainly belonging to the protein part of the granule complex Sodium released Hi from the granules by equivalent ionic exchange at the storage sites, and Hi 5 HT and sodium competed for these sites (Uvnas Åborg and Bergendorff 1970 Bergqvist Samuelson and Uvnas 1971 Bergendorff and Uvnas 1972)

Considering these observations it was not surprising to find that mast cell granules *in vitro* unselectively took up and stored organic and inorganic cations All the cations seem to be bound mainly at the same storage sites since all the uptake curves were similar and competition for uptake could be demonstrated

The affinities of the phenylethylamine derivatives for the storage sites are strikingly similar The K_m figures calculated from the uptake curves should not be taken as exact expressions of the strength with which the different amines are bound to the ionic sites However they still demonstrate that even rather considerable changes in the chemical structure — hydroxylation in the aromatic ring and/or hydroxylation in the diethylamine side chain do not significantly alter the affinities of the amines for the granule storage sites

The above observations agree with the assumption that the storage of catechol amines and related amines is primarily dependent on the presence of an ionized amino group and not or to only a minor degree on hydrogen bonds or other intermolecular electrostatic forces as proposed by Green (1967) and several others *In* all the amines investigated the primary amino group can be considered to be fully ionized at pH 7 hence their identical affinities for the granule binding sites

Trp A 5 HT and Hi have somewhat higher affinities for the storage sites than the phenylethylamine derivatives The reason for this higher affinity is obscure but it might be due to the fact that they are diamines The heterocyclic amino group which is slightly ionized at pH 7 might add to the electrostatic binding forces

The unselective uptake of biogenic amines by mast cell granules *in vitro* contrasts sharply with the selective storage of amines *in vivo* Rat peritoneal mast cell granules *in vivo* store mainly Hi with a small admixture of 5 HT (ratio Hi/5 HT = 30/1)

When considering these differences between *in vitro* and *in vivo* conditions it has to be remembered that mast cell granules isolated from water lysed mast cells have no surrounding membrane and thus *in vitro* there is no structure to interfere with the ionic exchange between the granule storage sites and cations in the extra granular medium

Rat mast cells *in vitro* are able to take up from a suspension medium and store in their granules not only the endogenous amines Hi and 5 HT but also DA and NA (Cabut and Haegermark 1966 Heisler and Uvnas 1972 Frisk Holmberg 1972) Furthermore *in vitro* incubation of rat mast cells in media containing histidine 5 hydroxytryptophane or dopa results in the formation of the respective amine and its storage in the granules (Schayer 1956 Lagunoff and Benditt 1959, Furano and Green 1964 Slorach and Uvnas 1968) However, the cells are not able to form 5 HT from tryptophan or NA from dopa since they lack the requisite hydroxylases (Slorach and Uvnas 1968)

The examples given above indicate that the selectivity of the amine storage of the mast cell does not depend on the properties of the granule storage mechanism but on the availability of extracellular amines and their precursors and on the capability of the cells to transform these precursors to their respective amines

In previous articles we have described the Hi and 5 HT release from degranulating mast cells as being due to a cation exchange between granule stored Hi and 5 HT on the one hand and sodium in the extracellular fluid on the other (Thon and Uvnas 1967 Rohlich Anderson and Uvnas 1971 Bergendorff and Uvnas 1972) The high affinity of Ca ions for the granule binding sites (about 20 times higher than that of sodium indicates that on degranulation of mast cells the Ca ions present in the extracellular fluid (around 1 mM) suffice for a considerable release of histamine from the granule binding sites (the histamine releasing effect of various inorganic cations is shown in Fig 6)

In the present experiments many examples have been given of the unspecificity on the granule storage mechanism From the results one can envisage the uptake storage and release of granule amines as being simple ionic exchange processes Endogenous and exogenous amines as well as inorganic cations compete for common binding sites in the granule matrix

The weak cation binding properties of the granule heparin—protein complex forms the basis for the amine storage mechanism operating in the mast cells This fact is of special interest in view of our recent findings indicating the presence of cation binding sulphomucopolysaccharide (SMPS) protein complexes in other Hi and 5 HT stores as well as in nervous transmitter stores (Åborg Uvnas 1971 Fillion Nosal and Uvnas 1971) It is interesting to speculate about the significance of this general distribution of SMPS protein complexes in biogenic amine stores The amine storage mechanism of the mast cell may form a model valid for the storage of other releasable biogenic amines

This investigation was supported by Swedish Medical Research Council Grant B72 14X 39 08

References

ABORG C H and B UVNAS A mucopolysaccharide protein complex with amine binding properties in rat thrombocytes *Acta physiol scand* 1971 *81* 568—570

BERGENDORFF A and B UVNAS Storage of 5 Hydroxytryptamine in rat mast cells Evidence for an ionic binding to carboxyl groups in a granule heparin protein complex *Acta physiol scand* 1972 *84* 320—331

BERGQVIST U G SAMUELSSON and B UVNAS Chemical composition of basophil granules from isolated rat mast cells *Acta physiol scand* 1971 *83* 362—372

CABUT M and O HAEGERMARK Uptake storages and release of histamine by rat peritoneal mast cells *in vitro* *Acta physiol scand* 1966 *68* 206—214

FILLION G R NOSAL and B UVNAS The presence of a sulphomucopolysaccharide protein complex in adrenal medullary cell granules *Acta physiol scand* 1971 *83* 286—288

FRISK HOLMBERG M and B UVNAS The influence of chlorpromazine on the uptake of biogenic amines by rat mast cells *in vitro* *Acta physiol scand* 1972 *86* 1—11

FURANO A V and J P GREEN Uptake of biogenic amines by mast cells of the rat *J Physiol* (Lond) 1964 *170* 263—271

GREEN J P Uptake and binding of histamine *Fed Proc* 1967 *26* 211—218

HEISLER S and B UVNAS *In vitro* studies on the uptake of biogenic amines by rat mast cells *Acta physiol scand* 1972 *86* 145—154

LAGUNOFF D and E P BENDITT 5 hydroxytryptophan decarboxylase activity in rat mast cells *Amer J Physiol* 1959 *196* (5) 993—997

ROHLICH P P ANDERSON and B UVNAS Electron microscope observations on compound 48/80 induced degranulation in rat mast cells Evidence for sequential exocytosis of storage granules *J cell biol* 1971 *51* 465—483

SCHAYER, R W Formation and binding of histamine by free mast cells of rat peritoneal fluid *Amer J Physiol* 1956 *186* 199—202

SLORACH S A and B UVNAS Amine formation by rat mast cells *in vitro* *Acta physiol scand* 1968 *73* 457—470

THON I L and B UVNAS Mode of storage of histamine in mast cells *Acta physiol scand* 1966 *67* 455—470

THON I L and B UVNAS Degranulation and histamine release two consecutive steps in the response of rat mast cells to compound 48/80 *Acta physiol scand* 1967 *71* 303—315

UVNAS B C H ABORG and A BERGENDORFF Storage of histamine in mast cells Evidence for an ionic binding of histamine to protein carboxyls in the granule heparin protein complex *Acta physiol scand* 1970 *78* Suppl 336 1—26

Acte physiol scand 1973 87 223—227
From the Department of Physiology University of Lund Sweden

Degeneration Activity in the Rat Urinary Bladder

By

MATS ELMER

Received 9 May 1972

Abstract

ELMER M *Degeneration activity in the rat urinary bladder* Acta physiol scand 1973 87 223—227

Periods of increased activity were found in the urinary bladder of the rat in situ 50—90 h after postganglionic parasympathetic denervation The activity was not affected by hexamethonium eserine produced an increase of the activity and atropine and Hoechst 9980 totally abolished it It is concluded that the activity is due to transmitter leaking from degenerating post ganglionic nerve terminals and that the transmitter probably is acetylcholine

During the first few days after section of postganglionic autonomic nerves activity in the effector organ has been described This activity is due to a leakage of trans mitter from the degenerating nerve terminals (Emmelin and Trendelenburg 1972) In the parasympathetic system the postganglionic neurone is usually so short that the number of preparations in which the phenomenon can be studied is limited and it has hitherto almost solely been studied in salivary glands The urinary bladder of the rat however receives axons from ganglia situated outside the organ which makes it possible to perform a total postganglionic denervation (Carpenter and Rubin 1967)

In the present investigation increased activity was found in the urinary bladder of the rat following postganglionic parasympathetic denervation In the submaxillary gland of the cat the so called degeneration secretion could sometimes be obtained only if the gland had been sensitized by earlier parasympathetic decentralization or sympathetic denervation (Emmelin 1962) The same was found to be true for the activity in the rat urinary bladder which could be demonstrated only when the bladder muscle had been sensitized by earlier sympathetic denervation or para sympathetic decentralization The sensitivity of the rat bladder to acetylcholine is increased 2—3 weeks after sympathetic or preganglionic parasympathetic denerva tion (Elmer unpublished observation) In preliminary experiments when para sympathetic denervation was performed without earlier sensitization no increase of activity could be detected

References

ABORG C H and B UVNÄS A mucopolysaccharide protein complex with amine binding properties in rat thrombocytes *Acta physiol scand* 1971 *81* 568—570

BERGENDORFF A and B UVNÄS Storage of 5 Hydroxytryptamine in rat mast cells Evidence for an ionic binding to carboxyl groups in a granule heparin protein complex *Acta physiol scand* 1972 *84* 320—331

BERGQVIST U G SAMUELSSON and B UVNÄS Chemical composition of basophil granules from isolated rat mast cells *Acta physiol scand* 1971 *83* 362—372

CABUT M and Ö HAEGERMARK, Uptake storages and release of histamine by rat peritoneal mast cells *in vitro* *Acta physiol scand* 1966 *68* 206—214

FILLION G R, NOSAL and B UVNÄS The presence of a sulphomucopolysaccharide protein complex in adrenal medullary cell granules *Acta physiol scand* 1971 *83* 286—288

FRISK HOLMBERG M and B UVNÄS The influence of chlorpromazine on the uptake of biogenic amines by rat mast cells *in vitro* *Acta physiol scand* 1972 *86* 1—11

FURANO A V and J P GREEN Uptake of biogenic amines by mast cells of the rat *J Physiol* (Lond) 1964 *170* 263—271

GREEN J P Uptake and binding of histamine *Fed Proc* 1967 *26* 211—218

HEISLER S and B UVNÄS *In vitro* studies on the uptake of biogenic amines by rat mast cells *Acta physiol scand* 1972 *86* 145—154

LAGUNOFF D and E P BENDITT 5 hydroxytryptophan decarboxylase activity in rat mast cells *Amer J Physiol* 1959 *196* (5) 993—997

ROHLICH P P ANDERSON and B UVNÄS Electron microscope observations on compound 48/80-induced degranulation in rat mast cells Evidence for sequential exocytosis of storage granules *J cell biol* 1971 *51* 465—483

SCHAYER, R W Formation and binding of histamine by free mast cells of rat peritoneal fluid *Amer J Physiol* 1956 *186* 199—202

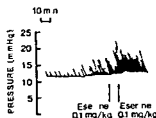
SLORACH S A and B UVNÄS Amine formation by rat mast cells *in vitro* *Acta physiol scand* 1968 *73* 457—470

THON I L and B UVNÄS Mode of storage of histamine in mast cells *Acta physiol scand* 1966 *67* 455—470

THON I L and B UVNÄS Degranulation and histamine release two consecutive steps in the response of rat mast cells to compound 48/80 *Acta physiol scand* 1967 *71* 303—315

UVNÄS B C H ÅBORG and A BERGENDORFF Storage of histamine in mast cells Evidence for an ionic binding of histamine to protein carboxyls in the granule heparin protein complex *Acta physiol scand* 1970 *78* Suppl 336 1—26





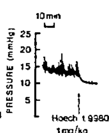


Fig 1 Fig 2 Fig 3

Fig 1 A period of increased activity in the urinary bladder of the rat starting 72 h after post ganglionic parasympathetic denervation

Fig 2 Effct of eserine on the increased activity in the urinary bladder of the rat

Fig 3 Effect of the parasympatholytic agent Hoechst 9980 on the increased activity in the urinary bladder of the rat

Effects of drugs

Hexamethonium The ganglionic blocking agent hexamethonium 10 mg/kg did not affect the periods of increased activity

Eserine When 0 1—0 2 mg/kg of eserine was injected during a period of activity both tone and contractions increased (Fig 2) Eserine had no effect on the bladder activity before the beginning of the periods of increased activity in the denervated preparation

Atropine 1 mg/kg of atropine totally abolished the contractions for 10—60 min when given during a period of increased activity but did not affect bladder tone After a higher dose of atropine (3 mg/kg) the inhibition of contractions lasted longer and the bladder tone decreased 1—2 mm Hg When atropine was given after eserine he inhibiting effect lasted for a shorter time than when the same dose of atropine was given without previous eserinization Atropine had no effect on the bladder activity of the control rats

Hoechst 9980 When a specific parasympatholytic agent Hoechst 9980 (Schaumann and Lindner 1951) was given in a dose of 1 mg/kg it both abolished the increased contractions and lowered bladder tone 2—3 mm Hg that is to the same level as in the resting bladder (Fig 3)

Chloralose A dose of 50 mg chloralose/kg when given during a period of increased activity sometimes caused a slight decrease in both contractions and tone which lasted for 10—30 min

Discussion

The activity of the urinary bladder of the rat after section of the pelvic nerves shows several similarities to the paroxysmal secretion of the parotid gland in cats after section of he postganglionic parasympathetic secretory nerve fibres in the auriculotemporal nerve (Emmelin and Stromblad 1958) This degen ration secretion from

the parotid gland starts and ceases earlier than the activity of the urinary bladder but it has about the same duration (24—36 h) and may appear in periods at irregular intervals in the same way It seems reasonable to conclude that the increased activity in the urinary bladder after postganglionic parasympathetic denervation is a degeneration activity

The anesthetic influences the degeneration activity in the bladder as is the case for salivary secretion When an extra dose of chloralose was given during a period of activity a slight decrease in both contractions and tone was sometimes seen

Physiological and pharmacological evidence indicates that the rat bladder in contrast to those of other species is devoid of intramural ganglion cells (Vanov 1965 Carpenter and Rand 1965) and these observations have been confirmed histologically (Carpenter and Rubin 1967 Chesher 1967) In the present study accordingly the ganglionic blocking agent hexamethonium had no effect on bladder activity after extirpation of the pelvic ganglia located outside the bladder wall on the lateral surface of the prostate gland

Bladder activity produced by electrical stimulation of the vesical nerves is potentiated by anticholinesterases (Carpenter 1963 Vanov 1965 Chesher 1970) This is true also for degeneration activity, as shown in the present study, which indicates that the transmitter supposed to leak from the degenerating terminals is aceteylcholine

The excitatory effect of acetylcholine on the bladder is antagonized by atropine while the response to stimulation of the parasympathetic nerves is almost completely atropine resistant (Langley and Andersson 1895) Several authors agree upon the atropine resistance of detrusor contractions elicited by parasympathetic stimulation but different theories have been suggested to explain this anomaly It has been proposed that the motor nerves of the bladder are non cholinergic in the dog (Henderson and Roepke 1934) in the guinea pig rat and rabbit (Ambache and Zar 1970) and in the guinea pig and rat (Dumsday 1971 Burnstock *et al* 1972) In the present investigation however the vesical response to the transmitter supposed to leak from the degenerating nerve endings could be totally abolished by atropine and the specific parasympatholytic agent Hoechst 9980 which indicates that the transmitter is acetylcholine

Dale and Gaddum (1930) discussing other atropine resistant mechanisms suggest that acetylcholine is liberated by the nerve endings in such an intimate relationship to the receptor that atropine cannot prevent its access A similar theory is also proposed by Ursillo and Clark (1956) and Chesher (1970) who found both atropine-sensitive and atropine resistant components of the response to electrical stimulation in the urinary bladder of the dog rabbit and rat

The present findings support the proximity theory of Dale and Gaddum provided that the distance between nerve terminal and receptor in the urinary bladder increases during the second third and fourth day after denervation as is the case in somatomotor neuromuscular junctions (Reger 1959) The access of atropine to the receptor sites may thereby be facilitated during degeneration of the nerve

Huković Rand and Vanov (1965) suggest that the acetylcholine released by nerve impulses may reach high concentration locally, sufficient to surmount the blockade of the receptors by atropine This explanation of the atropine resistance to nerve stimulation is consistent with the present experiments since the amount of trans mitter leaking from the degenerating nerve endings is reasonably maller than that liberated by electrical stimulation of the intact nerve The finding that the atropine blockade lasted for a shorter time after esernization also supports this theory of a competition for the receptors between acetylcholine and atropine

References

AMBACHE N and M ABOO ZAR Non-cholinergic transmission by postganglionic motor neurones in the mammalian bladder *J Physiol* (Lond) 1970 210 761—783

BURNSTOCK G B DUMSDAY and A SMYTHE Atropine resistant excitation of the urinary bladder the possibility of transmission via nerves releasing a purine nucleotide *Brit J Pharmacol* 1972 *44* 451—461

CARPENTER F G Excitation of rat urinary bladder by coaxial electrodes and by chemical agents *Amer J Physiol* 1963 *204* 727—731

CARPENTER F G and S A RAND Relation of acetylcholine release to response of the rat urinary bladder *J Physiol* (Lond) 1965 *180* 371—382

CARPENTER F G and R M RUBIN The motor innervation of the rat urinary bladder *J Physiol* (Lond) 1967 *192* 609—617

CHESHER G B Acetylcholine in extracts and perfusates of urinary blader *J Pharm Pharmacol* 1967 *19* 445—455

CHESHER G B Differentiation of receptors for exogenous and endogenous acetylcholine in the urinary bladder *Agents and Actions* 1970 *1* 128—132

DALE H H and J H GADDUM Reactions of denervated voluntary muscle and their bearing on the mode of action of parasympathetic and related nerves *J Physiol* (Lond) 1930 *70* 109—144

DUMSDAY B Atropine resistance of the urinary bladder innervation *J Pharm Pharmacol* 1971 32 222—225

EMMELIN N Submaxillary and sublingual secretion in cats during degeneration of post ganglionic parasympathetic fibres *J Physiol* (Lond) 1962 *162* 2 0—281

EMMELIN N and B C R STROMBLAD A "paroxysmal" secretion of saliva following parasympathetic denervation of the parotid gland *J Physiol* (Lond) 1958 *143* 506—514

EMMELIN N and U TRENDELENBURG Degeneration activity after parasympathetic or sympathetic denervation *Ergebn Physiol* 1972 In press

HENDERSON V E and M H ROEPKE The role of acetylcholine in bladder contractile mechanisms and in parasympathetic ganglia *J Pharmacol exp Ther* 1934 *51* 97—111

HUKOVIĆ S M J RAND and S VANOV Observations of an isolated innervated preparation of rat urinary bladder *Brit J Pharmacol* 1965 *24* 178—188

LANGLEY J N and H K ANDERSON The innervation of the pelvic and adjoining viscera Part II The bladder *J Physiol* (Lond) 1895 19 71—84

LANGWORTHY O R Innervation of the pelvic organs of the rat *Invest Urol* 1965 2 491—511

REGER J F Studies on the fine structure of normal and denervated neuromuscular junctions from mouse gastrocnemius *J Ultrast u t Res* 1959 2 269—282

SCHAUMANN O and E LINDNER Neue synthetische Verbindungen der Polamidonreihe mit parasympathicolytischer Wirkung *Naunyn—Schmiedeb rg's Arch exp Path Pharmak* 1951 *214* 93—102

URSILLO R C and B B CLARK The action of atropine on the urinary bladder of the dog and on the isolated nerve bladder strip preparation of the rabbit *J Pharmacol exp Ther* 1956 *118* 338—347

VANOV S Responses of the rat urinary bladder in situ to drugs and to nerve stimulation *Brit J Pharmacol* 1965 *24* 591—600

the parotid gland starts and ceases earlier than the activity of the urinary bladder but it has about the same duration (24—36 h) and may appear in periods at ir regular intervals in the same way It seems reasonable to conclude that the increased activity in the urinary bladder after postganglionic parasympathetic denervation is a degeneration activity

The anesthetic influences the degeneration activity in the bladder as is the case for salivary secretion When an extra dose of chloralose was given during a period of activity, a slight decrease in both contractions and tone was sometimes seen

Physiological and pharmacological evidence indicates that the rat bladder in contrast to those of other species is devoid of intramural ganglion cells (Vanov 1965 Carpenter and Rand 1965) and these observations have been confirmed histologically (Carpenter and Rubin 1967 Chesher 1967) In the present study accordingly the ganglionic blocking agent hexamethonium had no effect on bladder activity after extirpation of the pelvic ganglia located outside the bladder wall on the lateral surface of the prostate gland

Bladder activity produced by electrical stimulation of the vesical nerves is potentiated by anticholinesterases (Carpenter 1963 Vanov 1965 Chesher 1970) This is true also for degeneration activity as shown in the present study which indicates that the transmitter supposed to leak from the degenerating terminals is acetylcholine

The excitatory effect of acetylcholine on the bladder is antagonized by atropine, while the response to stimulation of the parasympathetic nerves is almost completely atropine resistant (Langley and Andersson 1895) Several authors agree upon the atropine resistance of detrusor contractions elicited by parasympathetic stimulation but different theories have been suggested to explain this anomaly It has been proposed that the motor nerves of the bladder are non cholinergic in the dog (Henderson and Roepke 1934) in the guinea pig rat and rabbit (Ambache and Zar 1970) and in the guinea pig and rat (Dumsday 1971 Burnstock et al 1972) In the present investigation however the vesical response to the transmitter supposed to leak from the degenerating nerve endings could be totally abolished by atropine and the specific parasympatholytic agent Hoechst 9980 which indicates that the transmitter is acetylcholine

Dale and Gaddum (1930) discussing other atropine resistant mechanisms suggest that acetylcholine is liberated by the nerve endings in such an intimate relationship to the receptor that atropine cannot prevent its access A similar theory is also proposed by Ursillo and Clark (1956) and Chesher (1970) who found both atropine sensitive and atropine resistant components of the response to electrical stimulation in the urinary bladder of the dog rabbit and rat

The present findings support the proximity theory of Dale and Gaddum, provided that the distance between nerve terminal and receptor in the urinary bladder increases during the second third and fourth day after denervation as is the case in somatomotor neuromuscular junctions (Reger 1959) The access of atropine to the receptor sites may thereby be facilitated during degeneration of the nerve

The HL receptors seem to belong to the group of receptors called atrial receptors of type B. The LL receptors on the other hand have not been identified so far. They continuously discharge on hemorrhage and seemingly also at ordinary blood volume.

It may be expected that these receptors adapt to or that the effector mechanism is exhausted on intensive and continuous stimulation as at maintained hypotension. If this is the case the circulatory adjustment on hemorrhage would be impaired. The effect would also be similar to the effect of exclusion of vagal afferents from the low pressure side.

This supposition has been examined in a series of experiments and the results indicate that the LL receptors adapt to and/or the elicited effect is exhausted on repeated bleeding to a controlled blood pressure level. This may play an essential role in the progress of oligemia to irreversible shock.

Material and Methods

Rats of the Sprague Dawley strain with a b.w. of 200—325 g were used. The rats were anesthetized with pentobarbitone (5—8 mg/100 g b.w.) i.p. No further anesthetic was given during the bleeding experiments. In the experiments on the effect of hemorrhage to a constant arterial pressure animals of the same breed were subjected alternatively to complete or partial deafferentation or were taken as controls.

The procedure and technique employed were the same as described earlier (Castenfors and Sjöstrand 1972, Castenfors *et al.* 1973). Heparin was used to prevent coagulation.

Results

Bleeding and reinfusion of blood to a controlled blood pressure

Fig. 1 shows an experiment in a rat with repeated drawing off and reinfusion of blood to maintain an arterial pressure level between 40 and 50 mm Hg. As seen from the figure the necessary volume drawn off each time decreases gradually until the critical point is reached when reinfusion has to be started. This appears after a loss of altogether 10 ml (about 60 % of the initial blood volume) 2 h after the first blood drawing. Small amounts are first infused to increase the blood pressure to 50 mm Hg. Later these infusions must be increased until the whole volume is reinfused. At this moment the animal dies in respiratory arrest.

From the record of the heart rate the following may be extracted. The heart rate decreases on the first blood drawings but increases somewhat on subsequent drawings simultanously with an increase of the level. The heart rate reaches almost the initial level briefly before the critical point.

The increase of the heart rate on drawing off blood when the mean level is increasing has been still more obvious in other animals.

Circulatory adjustment after reinfusion at the critical point

If the drawn blood volume is reinfused at the critical point the arterial pressure increases to the initial or somewhat higher or lower levels in different animal

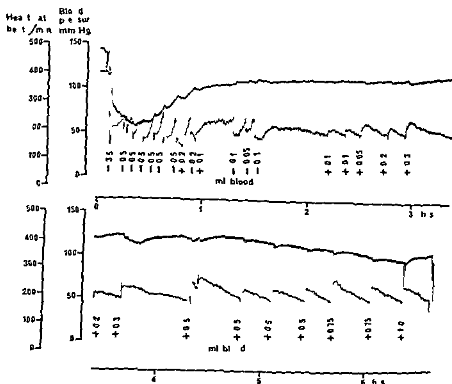


Fig 1 Heart rate on repeated blood drawings followed by infusions to maintain a pressure of 40—50 mm Hg in a Sprague Dawley rat Note that the heart rate decreases on the first blood drawings and that the mean level successively increases to the critical point when reinfusion starts Continuous recording

the level is stabilized or may gradually be lowered in some rats to circulatory insufficiency and death after 2—3 h In the latter case a transfusion of blood has only a transient effect (Fig 2)

Briefly after reinfusion at the critical point the resistance to bleeding is increased as seen from Fig 2 The loss of a given volume provokes first only a small decrease of the arterial pressure On repeated blood drawings after reinfusion the pressure drop increases and after about 1 h reaches the same level as before bleeding The interval up to this moment is in the following called the phase of increased resistance to bleeding In Fig 3 the arterial pressure on each blood drawing in the experiment demonstrated in Fig 2 is plotted against the time after the critical point in order to relate the changes of resistance to time On testing different equations the presented exponential function has been found to tally best with the experimental data

If repeated blood drawings to a constant arterial pressure are performed once again after this phase the critical point is reached much earlier and after a smaller blood loss than in the first period (Fig 2 and 4 and Table I) It emerges also from Fig 2 and 4 that the heart rate increases with the pressure drop and then decreases somewhat to the critical point Furthermore the heart rate decreases on reinfusion

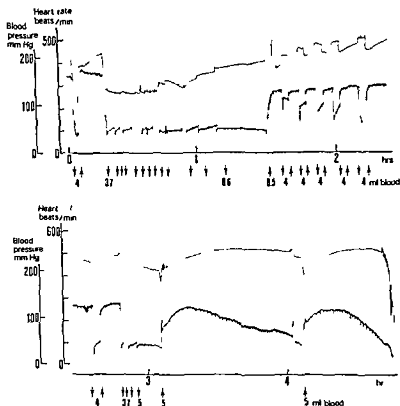


Fig 2 Repeated blood loss to maintained arterial pressure of 40—50 mm Hg followed by reinfusion at the critical point in a Sprague Dawley rat Briefly after reinfusion 4 ml blood is drawn off and reinfused repeatedly /0 min later when the response to the blood loss is about the same as initially bleeding to the critical point is renewed After the subsequent reinfusion the arterial pressure is first restored but then falls successively Transfusion of 5 ml blood provokes only a transient restitution The animal dies 40 min later Note the change of the heart rate re ponse on bleeding after the fir t bleeding period Continuous time cale

Thus the heart rate changes inversely as during the first period It is obvious from experiments of this kind that the phase of increased resistance to bleeding is followed by a period of decreased resistance This period lasts for hours Thus in one experiment the time of repeated bleeding to the critical point was markedly decreased 5 h after the reinfusion Also in this case the heart rate increased on blood loss Exclusion of the laryngeal pathway and subsequent vagotomy exerted no apparent effect on heart rate or the blood pressure response

The decreased resistance to bleeding after a blood loss to the critical point has also been demonstrated by the effect of a single blood drawing Fig 4 shows first the effect of drawing off and reinfusion of 20 % of the blood volume during the phase of increased resistance The same volume is then drawn 1 /2 h later

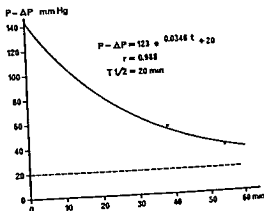


Fig 3 The arterial pressure after a blood loss of 4 ml as a measure of the resistance to bleeding is plotted against time after the reinfusion at the critical point in the the experiment demonstrated in Fig 2 The resistance falls off exponentially according to the equation given in the figure

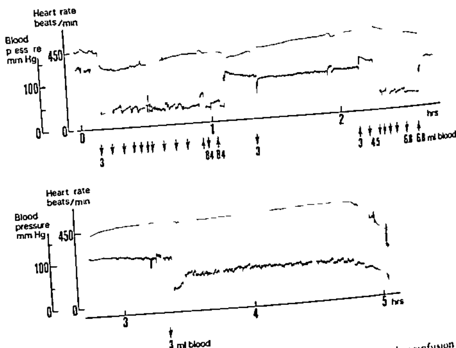


Fig 4 Repeated bleeding to an arterial pressure of 40—50 mm Hg with reinfusion at the critical point 15 min later 3 ml blood is drawn and reinfused 50 min later Renewed bleeding to the critical point and reinfusion Forty minutes later 3 ml blood is drawn off This is followed by incomplete restitution and a successive decrease of the arterial pressure to the death of the animal × = occlusion of the carotid arteries Note the larger blood volume drawn to reach 40 mm Hg (4 5 ml against 3 ml) and the more rapid restitution in each drawing during the second bleeding period as also the shorter interval to the critical point

TABLE I Time to critical point and total blood volume lost on first and second period of bleeding to a constant pressure level

	By g	1 st period min	Vol ml	2 nd period min	Vol ml	Remarks
	312	72	11.8	12	6.9	
	326	53	9.8	9	7.0	
	241	120	10.2	28	6.0	
	235	70	8.5	18	5.0	
	317	74	10.6	25	6.5	
	256	57	8.4	21	6.8	
	242	52	8.0	16	6.4	Lar deaff
Mean $\bar{x}$	276	71	9.6	18.4	6.4	
Mean S.E.	15.38	8.85	0.52	2.57	0.26	

The arterial pressure is first compensated to about 70 % of the initial level. Then the arterial pressure drops successively until the death of the animal 1 1/2 h. The drawn blood volume is only about a third of the total volume drawn during the first period with constant pressure.

The effect of complete or partial exclusion of vagal afferents on the time and amount of bleeding to the critical point

The afferents of the LL receptors run in varying pathways in differents animals. In most of the earlier studied rats the main pathway has been via the laryngeal communicans. In some animals the vagus trunk (inclusive a thin nerve probably the cardiac nerve which is easily separated after opening of the sheath and which enter the trunk at the nodose ganglion) seems to convey most of the afferents from these receptors. By cutting the laryngeal nerves and/or the supposed cardiac nerves and/or the vagal trunk alternatively a partial or complete exclusion of the afferents is obtained. This has been done in a number of rats to study the effect on the resistance to bleeding.

The time interval to the critical point and the total blood volume drawn off are shown in Table II and III. It emerges from these tables that the bleeding time to the critical point varies to a high degree in different animals but is significantly shorter after partial and especially after complete vagal deafferentation. This is also demonstrated by Fig. 2, 4 and 5. The total drawn volume is on an average 9 % smaller on partial or complete deafferentation. It has also been evident from these experiments that the first drawing necessary to reach a pressure just below 40 mm Hg is generally larger after partial or complete deafferentation. Thus this volume in per cent of the total drawn volume shows an inverse relation to the time interval to the critical point for the whole material ($r = -0.6$). On the other hand the initial blood loss shows no correlation to the total blood volume. The conditions indicate that the larger the resistance to a sudden blood loss the shorter is the time to the critical point. This is evident also from one especially pronounced case. In the ex

TABLE II Bleeding time to the critical point and total bled volume in controls and after partial or complete vagal deafferentation

	B w g	Sex	Blood vol ml	Bleed time min	Blood lost ml		Remarks
Controls	326	male	17.9	52	9.8	55	
	340		18.7	80	11.0	60	
	235		12.9	70	8.5	66	
	241		13.2	120	10.2	77	
	246		13.3	93	8.9	66	
	308		16.9	85	12.0	71	
	246		13.5	84	8.5	63	
	313		17.1	116	11.1	65	
	260	female	14.3	82	7.6	52	
	246		13.5	72	6.8	50	
Mean $\bar{x}$	276		15.2	85	9.4	63	
S E	12.87		0.71	6.47	0.53	2.67	
Partial deaff	242	male	13.3	50	8.0	60	l d
	335		18.2	62	11.0	60	l d
	260		15.4	67	9.4	61	l d
	283		15.5	44	6.9	45	l d + l v
	295		16.2	58	9.3	57	l d + r v
	326		17.9	56	10.0	60	l d + c n n
	246		13.5	64	8.6	64	l d + c n n
	267	female	14.7	32	6.4	44	l d
	223		12.2	56	5.6	46	l d + l v
	283	male	15.5	44	9.6	46	v b
	323		17.7	51	8.0	45	v t
	307		16.9	76	9.9	59	v t
	280		15.4	67	9.4	61	l d + v t
	246		13.5	40	7.7	57	v t
	244	female	13.4	40	7.0	[illegible]	l d + v t
Mean $\bar{x}$	279		15.3	54	8.5	54	
S E	8.84		0.48	3.17	0.39	1.88	
Complete deaff	245	male	13.5	16	8.0	60	
	273		15.0	37	7.2	48	
	214		11.8	32	6.0	51	
	307		16.9	49	10.2	60	
	269		14.8	35	9.5	64	
	265		14.6	43	8.0	[illegible]	
	246	female	13.5	24	6.4	48	
	245		13.5	36	[illegible]	[illegible]	
Mean $\bar{x}$	258		14.2	34	7.7	54	
S E	9.65		0.53	3.65	0.54	2.33	

Abbreviations
l d = laryngeal deafferentation l v = left vagus cut
r v = right vagus cut c n n = cardiac nerves cut
v b = vagus cut bilaterally v t = vagus trunk cut bilaterally with cardiac nerves intact

periment 60 % of the blood volume has been drawn to lower the pressure to about 50 mm Hg after complete deafferentation Then the arterial pressure drops successively during 16 minutes to 40 mm Hg (Fig 6) The same figure demonstrates also the effect on heart rate and blood pressure on drawing off a given volume after laryngeal deafferentation and vagotomy respectively Only some of the afferents from the LL receptors obviously run via the laryngeal communicans in this case

TABLE III Differences (d) between group means of individual data presented in Table II A Control rats versus completely deafferentated rats B Control rats versus partially deafferentated rats C Partially deafferentated versus completely deafferentated rats

		B w g	Blood vol ml	Bleed time min	Blood lost ml	
A)	f	16	16	16	16	16
	d	18	1 0	51	1 ,*	9**
B)	f	23	23	23	23	23
	d	−3	−0 1	31	0 9	9
C)	f	21	21	21	21	21
	d	21	1 1	20**	0 8	±0

f = degrees of freedom
* = level of significance

The bleeding time to the critical point has been correlated to the time of heart rate depression on blood loss in the controls and in the animals with partial deafferentation The duration of the heart rate depression has been calculated from the start of the first blood drawing to the time when the heart rate has reached the initial level or a constant level below this level The results are presented in Fig 7 There is a direct correlation which implies that the critical point is reached simultaneously with the disappearance of the vagal effect on the heart elicited on the initial blood loss The disappearance time seems also to be correlated to the initial decrease of the heart rate which means that the size of the cardiac depression which can be expressed by the area below a line drawn from the initial heart rate level to the level at the critical point is roughly correlated to the time of restitution and to the lost blood volume

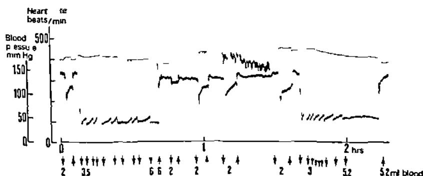


Fig 5 Two periods of hemorrhage to the critical point in a Sprague Dawley rat after complete vagal deafferentation Note the increased heart rate on the first blood drawing the less pronounced period of increased resistance to bleeding after the first period and that the second period lasts almost as long as the first The total drawn volume is also practically the same compare with Fig 7 and 4 The heart rate recording is artificially disturbed between 70 and 100 minutes on the time scale

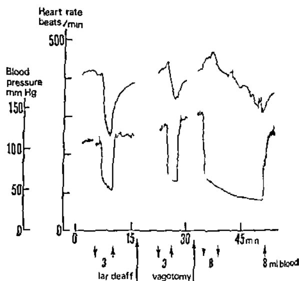
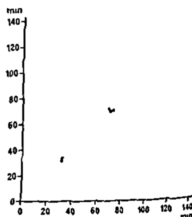


Fig 6 Changes of heart rate and arterial pressure on drawing off 3 ml blood in a Sprague Dawley rat before and after laryngeal de afferentation After a subsequent complete vagotomy 8 ml (about 60 % of total blood volume) is drawn off to reach the same pressure level as after 3 ml be fore The arterial pressure then falls gradually



ig 7 Correlation between time to the critical point ordinate) and duration of heart rate depression abscissa) in controls and partially deafferentated nimals y = 0 86 x − 5 47 r = 0 95

If blood is drawn after the reinfusion at the critical point in animals with com plete deafferentation it is shown that the phase of increased resistance to bleeding is shorter and less pronounced than in the controls Furthermore on a renewed period of repeated blood drawings to the critical point after this phase the time and amount of bleeding on the whole is the same as in the first period (Fig 5)

The relationship between the initial depression of the heart rate on hemorrhage as also the initially drawn blood volume and the time to the critical point has made it possible roughly to predict the effect of partial deafferentation This has been applied in some of the experiments When laryngeal deafferentation has provoked only a small effect on a subsequent blood loss it has been combined with cutting the right or left vagus nerve or the supposed cardiac nerves on both sides This explains the varying procedures indicated in Table II

In 7 expts (3 not included in Table II) only the laryngeal pathway has been excluded The mean time to the critical point in this series is 48 min (range 20—67) and for the total drawn blood volume 56 % (range 48—61) of the total blood volume In none of these animals was the bradycardia on hemorrhage completely annulled by the deafferentation as in most of the earlier examined series of rats It emerges from Table II that cutting the vagal trunk on one or both sides has not a more pronounced effect than exclusion of the laryngeal nerves alone or in combination with the cardiac nerves

Most of the experiments were performed on male rats but there seems to be no difference between male and female rats concerning the time to the critical point However the drawn blood volume is smaller in the female rats This may be related to the lower hemoglobin concentration in female rats (Gemzell and Sjostrand 1956)

Discussion

The results of this paper are in keeping with the earlier proposed concept that vagal afferents play an essential part in the restitution of the arterial pressure on a blood loss The susceptibility to bleeding has earlier been found to be increased on laryngeal deafferentation and/or cutting the vagal trunk (Castenfors *et al* 1972 b) The experiments with repeated bleeding to the critical point with and without vagal deafferentation confirm these observations It has been assumed that there are receptors on the low pressure side (LL receptors) which influence the tone of the capacity vessels on a decrease of the blood volume The same receptor mechanism seems to counteract the effect of the release of the tonic effect of the arterial baroreceptor mechanism as well as the sympathetic action elicited by cerebral ischemia on bleeding (Castenfors and Sjostrand 1973)

To explain the combination of a parasympathetic influence on the heart with an increased tone of the capacity vessels the hypothesis has been advanced that the latter effect is mediated by a stimulation of the hypophysis to release of ADH vasopressin in concentrations large enough to act on the capacity vessels (Castenfors *et al* 1973) This concept is supported by the present observation that just after the critical point when the concentration of ADH vasopressin in the blood and tissues would be high there appears a phase of increased resistance to bleeding disappearing exponentially The half time 20 min corresponds to the appearance of the late phase subsequent to an exclusion of the laryngeal pathway under normal conditions and after hemorrhage (Castenfors *et al* 1973) An effect of ADH on the resistance to hemorrhage has been postulated earlier (Frieden *et al* 1954 Weinstein *et al* 1960 Rocha e Silva and Rosenberg 1969)

The reported observations indicate in addition that the LL receptors adapt on bleeding to maintained low arterial pressure This is shown by a change of the heart rate response to drawing off blood and by the successive increase of the heart rate The intimate relation between the duration of the effect on heart rate and the bleeding time to the critical point indicates moreover an intimate association between the parasympathetic action and the effect on the capacity vessels

The long duration of the period of decreased resistance to bleeding after the critical point raises the question whether the efferent part of the regulatory mechanism also is put out of function This question is being submitted to further experiments Whatever may be the answer it seems obvious that a prolonged period of hypotension inhibits the effect of the LL receptors and provokes conditions similar to vagal deafferentation Thus the blood pressure restitution on a sudden blood loss is more rapid and the tone on the heart is changed in a sympathetic direction simultaneously with a lowering of the resistance to bleeding

It is tempting to explain the effects of vagal deafferentation and bleeding to the critical point by assuming two different regulatory systems acting in the circulatory adjustment on bleeding one elicited by a decrease of the effect of the arterial baroreceptors and by the cerebral ischemic reflex and acting via the sympathetic nervous system predominantly on the resistance vessels the other elicited by receptors on the low pressure side mediated by vagal afferents which changes the tone on the heart in a parasympathetic direction and by possible stimulation of the hypophysis to an increased release of ADH vasopressin acts predominantly on the capacity vessels The first system adjusts first and foremost the blood pressure and the second the distribution of the blood between the peripheral capacity vessels and the central vessels Tentatively the 2 systems may be called the pressure regulating system (PRS) and the volume regulating system (VRS) Ordinarily the circulatory adjustment on a blood loss is effected by the cooperation between the two systems The PRS effect the instantaneous adjustment of the arterial pressure but is rather rapidly exhausted at low pressure The VRS successively takes over the principal part of the restitution and thereby the PRS may be free for subsequent adjustments On exclusion of the VRS by vagal deafferentation or prolonged hypotension the circulatory control is impaired Thus circulatory insufficiency and death may appear spontaneously or after a moderate blood loss

The earlier observations of a transient restitution of the arterial pressure after a moderate blood loss preceded or followed by vagal deafferentation (*Castenfors et al* 1973) are also explained by this concept of two regulatory systems Thus on exclusion of VRS by deafferentation the arterial pressure is restored solely by PRS and thus more rapidly However as a result of its adaptation or exhaustion the restitution is only temporary and followed by circulatory insufficiency and death

The reported observations of the effect of oligemic hypotension in the rat are similar to those described in numerous papers on experiments in dogs (see Wiggers 1950) The stage before reinfusion after hemorrhage has been called the impending stage after which the reinfusion may be followed by either maintained restitution or irreversible shock It has been suggested that the progress to shock is effected by the decrease of the tissue perfusion as a result of the increased tone of the resistance vessels and by dilatation of the peripheral vessels on the venous side possibly provoked by toxic substances (see Fine 1965) The results of this series of investigations on the effect of vagal afferents on circulatory control in the rat indicate that the change from oligemia to progressive irreversible shock appears on the adaptation

or exhaustion of the volume regulation. The favourable effect of inhibition of the sympathetic activity on hemorrhage is well documented in several investigations on the effect of sympathetic blockade or denervation. The exclusion of the ADH vasopressin relase on maintained hypotension may explain the appearance of decreased vascular tone of the peripheral capacity vessels described by some authors (Zweifach and Thomas 1954, Alexander 1955). Thus the exclusion of the favourable effect of VSR on tissue perfusion and on the tone of the capacity vessels would result in a progressive disturbance of the circulation, which is not relieved by measures additionally increasing the tone of the resistance vessels or, at the progressive stage, blood infusions filling up further the atonic capacity vessels.

References

ALEXANDER, R. S. Venomotor tone in hemorrhage and schock. *Circulat. Res.* 1955. *3*. 181—190.

CASTENFORS, J. and T. SJÖSTRAND. Circulatory control via nagal afferents. I. Adjustment of heart rate to variations of blood volume in the rat. *Acta physiol. scand.* 1972. *84*. 347—354.

CASTENFORS, J. and T. SJÖSTRAND. Circulatory control via vagal afferents. IV. Integration of the central control mechanisms in the circulatory adaptation to variations of blood volume. *Acta physiol. scand.* 1973. *87*. 188—198.

CASTENFORS, J., E. KNUTSSON and T. SJÖSTRAND. Circulatory control via vagal afferents. II. Effects of hemorrhage on afferent discharges via the laryngeal communicans in the rat. *Acta physiol. scand.* 1972. *84*. 355—365.

CASTENFORS, J., E. KNUTSSON and T. SJÖSTRAND. Circulatory control via vagal afferents. III. Control of arterial blood pressure by afferents via the laryngeal communicans with special regard to the restitution after hemorrhage. *Acta physiol. scand.* 1973. *87*. 176—187.

FINE, J. Shock and peripheral circulatory insufficiency. In: *Handbook of Physiology.* Washington D.C. 1965. p. 2037—2069.

FRIEDEN, J., A. D. KELLER, C. P. HICKMAN and J. H. WATERS. Decreased resistance to hemorrhage in neurohypophysectomized dog. *Circulat. Res.* 1954. 2. 214—220.

GENZELL, C. A. and T. SJÖSTRAND. The effects of the gonads on the total amount of hemoglobin and blood volume in the rat. *Acta Endocrinology* 1956. *21*. 86—96.

ROCHA E SILVA, M. JR. and M. ROSENBERG. The release of vasopressin in response to hemorrhage and its role in the mechanism of blood pressure regulation. *J. Physiol.* (Lond.) 1969. *202*. 535—557.

WEINSTEIN, H., R. M. BERNE and H. SACHS. Vasopressin in blood. Effect of hemorrhage. *Endocrinology* 1960. *66*. 712—718.

WIGGERS, C. J. *Physiology of shock.* Harvard University Press. Cambridge, Mass. 1950.

ZWEIFACH, B. W. and L. THOMAS. The relationship between the vascular manifestations of shock produced by endotoxin, trauma and hemorrhage. *J. exp. Med.* 1957. *106*. 385—401.

Acta physiol scand 1973 87 240—245

From the Department of Medical Chemistry Royal Veterinary College Stockholm and the Burn Center Department of Plastic Surgery University Hospital Uppsala Sweden

Increased Synthesis of Prostaglandins in the Guinea Pig Following Scalding Injury

By

MATS HAMBERG and CARL EVERT JONSSON

Received 10 May 1972

Abstract

HAMBERG M and C E JONSSON *Increased synthesis of prostaglandins in the Guinea Pig following scalding injury* Acta physiol scand 1973 87 240—245

The urinary excretion in guinea pigs of the major urinary metabolite of prostaglandins E_1 and E_2 viz *5β 7α* dihydroxy 11 *ketotetranor* prostanoic acid was increased 5—9 fold following scalding injury Lipid extracts of homogenates of scalded guinea pig skin contained 20—40 times more smooth muscle stimulating activity than did extracts of non scalded skin Prostaglandins (mainly PGE and $PGF_{2\alpha}$) were responsible for about 60 % of this activity

Histamine serotonin and kinin forming activity have earlier been demonstrated in peripheral lymph of dogs exposed to burn injury (Edery and Lewis 1963) Recently the presence of smooth muscle stimulating lipids in lymph from scalded dog paws was reported (Jonsson 1971) It was subsequently shown that the major part of this material was identical with prostaglandin E (Änggard and Jonsson 1971)

In this communication we report increased urinary excretion of a prostaglandin metabolite as well as increased skin content of prostaglandins in guinea pigs exposed to scalding injury

Both biosynthesis and metabolism of prostaglandins have been studied in the guinea pig (Änggard and Samuelsson 1964 Änggard Green and Samuelsson 1965 Hamberg and Israelsson 1970 Hamberg and Samuelsson 1972) The major urinary metabolite of prostaglandins F_1 and F (PGL₁ and PGE) was identified as *5β 7α-dihydroxy* 11 ketotetranor prostanoic acid (Hamberg and Samuelsson 1969) Recently a method for quantitative determination of this metabolite was developed (Hamberg and Samuelsson 1972)

Materials and methods

Urinary excretion of PGE_1 and PGE_2 metabolite

Male guinea pigs (537—624 g) were kept immobilized in cages and given food and water *ad libitum* 24 h fractions of urine were collected quantitatively by a plastic tubing adapted to the penis Skin burns were produced under ether anesthesia by immersing part of the back of

the animals in 80 C water for 10 s. The scalded area was approximately 15 % of body area 5β 7α dihydroxy 11 ketotetranor prostanoic acid was determined by a method recently described (Hamberg and Samuelsson 1972) using gas-liquid chromatography in combination with mass spectrometry As carrier 2 4β 7β 8α tetradeutero-5β 7α-dihydroxy 11 ketotetranor prostanoic acid was used

Skin content of prostaglandins

The animals about 500 g were anesthetized with ether and the back was shaved One of the lateral sides of the back was immersed in water of 70 C for 10 s The other side was used as control 2 h later the animals were again anesthetized and skin from scalded and non scalded areas was excised and immediately transferred to absolute ethanol containing solid CO_2 Skin (wet weight 2—10 g) was minced with scissors on ice and homogenized at 0 C for 2 min in 5—10 ml absolute ethanol containing 0 1 % α tocopherol as antioxidant The homogenate was centrifuged at 4 C for 10 min. The supernatant was collected and the residue twice resuspended and centrifuged Dry weight of the residue was recorded after drying at 110 C for 48 h To the combined supernatants an equal volume of 0 9 % NaCl solution and tritium labelled PGE_1 (0 1 μCi specific activity 87 3 Ci/mmole smooth muscle stimulating activity equivalent to 0 1 ng PGE_2 New England Nuclear Corp) was added Lipid extracts were prepared as described by Unger Stamford and Bennett (1971) After evaporation of the organic solvents the dry residue was either reconstituted in the Jalon s soution with 10 % ethanol for bioassay or dissolved in organic solvents for chromatography

For silicic acid column and thin layer chromatography 0 2 μCi of ^3H PGE_1 (specific activity 87 3 Ci/mmole New England Nuclear Corp) and 0 2 μCi of ^3H PGF (specific activity 53 mCi/mmole smooth muscle stimulating activity equivalent to 6 ng PGE_2 Granstrom Inger and Samuelsson 1965) were added to ethanol extracts of burned skin homogenates

Column chromatography was performed essentially as described by Bygdeman and Samuelsson (1966) The silicic acid (Unisil 100—200 mesh Clarkson Chemical Corp Penns) was activated at 110 C The columns (1 g) were prepared with ethyl acetate benzene (1 9) and eluted by gradient elution with increasing concentrations of ethyl acetate starting with a ratio of 1 9 and ending with 100 % after which the columns were eluted with 15 ml methanol Fractions of 1 5 ml were collected Aliquots of the fractions were assayed for radioactivity Fractions of the chromatogram were pooled and assayed for biological activity

Thin layer chromatography was performed as described by Gréen and Samuelsson (1964) with some modifications The silicic acid (Silica Gel G Merck) was washed as described by Bygdeman and Samuelsson (1966) The extract was dissolved in ethanol and applied to the plate on a line with reference samples (PGE_1 and PGF_1) on both sides. After development in the A I system reference spots were visualized by spraying with phosphomolybdic acid Sections of about 1 cm were scraped off and extracted twice with ethanol The ethanol eluate was filtered evaporated and assayed for radioactivity and smooth muscle stimulating activity

For separation of individual prostaglandins thin layer chromatography was performed with silver nitrate impregnated plates (4 %) after group separation of prostaglandin E- and F-compounds by means of silicic acid column chromatography as described above The isolated peaks of radioactivity were applied and analyzed as described above Moving phase was A II (Green and Samuelsson 1964)

Bioassay was performed on the isolated colon of the gerbil (Weeks Schultz and Brown 1968) Usually 3 point assays were performed Smooth muscle stimulating activity is expressed in terms of PGE_2 Organic solvents of fractions to be assayed were evaporated *in vacuo* under reduced pressure or under a stream of N_2 and the dry residue dissolved in de Jalon s solution with 10 % ethanol

The response of the gerbil colon for different prostaglandins relative to that of PGE_2 (taken as 1) was approximately estimated to PGE_1 0 3 $PGF_{2α}$ 0 01 PGF 0 1—0 5 (*cf* Ramwell and Shaw 1966 Karim and Hillier 1968 Weeks *et al* 1968)

Results

The scalding injury in the 2 sets of experiments resulted in a deep second degree burn At 2 h after scalding a local edema had formed and wet weight was about twice that in non scalded skin Later a firm scar formed which was partly covered with hair (*cf* Douglas and Jonsson 1969)

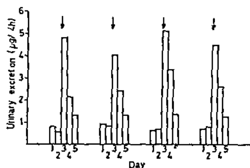


Fig 1

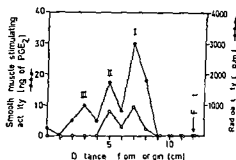


Fig 2

Fig 1 Daily urinary excretion of 5β 7α dihydroxy 11 ketotetranor prostanoic acid before and after scalding injury Arrows indicate time of scalding

Fig 2 Distribution of smooth muscle stimulating activity of thin layer chromatography of lipid extract obtained from scalded skin (wet weight 10 g) The lipid extract contained biological activity equivalent to 1,0 ng PGE_2 After development sections were assayed for biological activity on colon of the gerbil and radioactivity due to the added labelled PGE_1 and PGF Smooth muscle stimulating activity due to these have been subtracted

During the 24 h period following the scalding injury there was a 5 to 9 fold increase in urinary excretion of 5β 7α dihydroxy 11 ketotetranor prostanoic acid (Fig 1) Also during the 2 following 24 h periods the excretion was increased

As a rough estimate of the prostaglandin content in skin lipid extracts were prepared and assayed for biological activity on the gerbil colon which is known to be sensitive for prostaglandins Extracts from scalded skin contained 20—40 times more smooth muscle stimulating activity than did extracts from non scalded skin (Table I)

On thin layer chromatography of the lipid extracts with tritium labelled PGE_1 and PGF_1 as references 3 peaks of smooth muscle stimulating activity appeared (Fig 2) Peaks I and II co chromatographed with PGE_1 (R_F ~ 0 69) and PGF_1 (R_F = 0 53) respectively Also on silicic acid column chromatography 3 peaks of biological activity appeared one peak with the expected elution volume of a prostaglandin E compound and one with that of a prostaglandin F compound (Samuelsson *et al* 1963) Both biological peaks co-chromatographed with added radioactive tracers (^3H PGE_1 and ^3H $PGF_{1\alpha}$ respectively) In addition to these peaks material with tachyphylactic properties on gerbil colon was eluted with ethyl acetate and methanol from the silicic acid columns

As recorded on colon of the gerbil it was estimated that prostaglandins accounted for 80 % of the biological activity of acidic lipid extracts

The 2 chromatographic procedures mentioned do not allow separation between the individual PGE and PGF compounds Therefore thin layer chromatography with silver nitrate impregnated silica gel was carried out This showed that the major parts of the smooth muscle stimulating material in peaks I and II were identical with PGE and PGF_2 respectively However small amounts of smooth

TABLE 1 Smooth muscle stimulating activity in lipid extracts of scalded and non scalded skin. Biological activity is expressed in terms of PGE_1 (ng/g dry weight) after corrections for losses due to extraction

Exp no	Scalded	Non scalded
1	220	10
2	170	5
3	430	10
4	100	4
Mean	230	7

muscle stimulating activity were present at the positions of PGE_1 and $PGF_{1\alpha}$. Material in peak III of the original thin layer chromatography or material eluted with ethyl acetate and methanol from the silic acid columns was not further characterized.

Discussion

The urinary excretion of 5β 7α dihydroxy 11 ketotetranor prostanoic acid before scalding injury was found to be 0 6—0 9 μg/24 h in the present study. These values are in agreement with values for the basal excretion of the metabolite earlier recorded (Hamberg and Samuelsson 1972). It has been shown (Hamberg and Samuelsson 1969) that about 28 % of administered tritium labelled PGE is converted into the urinary metabolite measured in this study. Accordingly the basal synthesis of PGE_1 and PGE found in the present study was 2—3 μg/24 h. After scalding injury there was a 5—9 fold increase in the amount of the metabolite in the first 24 h period (Fig 1). This corresponded to an increase in the basal synthesis of PGE_1 and PGE by 11—16 μg/24 h.

Results from biological assays of extracts from scalded skin also demonstrated an increased synthesis of prostaglandins not only of E compounds but also of F-compounds. Conclusions as to which extent the increased biosynthesis of prostaglandins is directed towards formation of E or F compounds cannot be drawn but results indicate that the two groups are formed in about equal amonts.

The events leading to the enhanced biosynthesis of prostaglandins in scalded guinea pig skin are not known at present. Prostaglandin biosynthesis has been demonstrated in skin from humans (Jonsson and Anggard 1972) and rat (Jouvenaz *et al* 1970, Ziboh and Hsia 1971). However the levels of endogenous prostaglandins are low as shown in human skin (Jonsson and Anggard 1972) and guinea pig skin (this paper). It is possible that the tissue damage resulting from the burn injury will lead to increased release of arachidonic acid and 8 11 14 eicosatrienoic acid precursors of PGE and PGF and PGE_1 and PGF_1 respectively from phospholipids and triglycerides. The availability of the free precursor acids in tissues containing prostaglandin synthetase is believed to be a major factor determining the rate of the biosynthesis of prostaglandins (Pace Asciak *et al* 1968).

The enhanced biosynthesis of prostaglandins in scalded tissue at least partly explains the increased excretion of the major urinary metabolite of PGE_1 and PGE after scalding injury However other sites of biosynthesis may also contribute. For instance the increased sympathetic activity accompanying a burn injury (Birke et al 1957) might lead to an increased synthesis of prostaglandins (Brundin 1968, Hedqvist 1970 Green and Samuelsson 1971) Further studies are needed to establish whether increased synthesis of prostaglandins following burn injury is of importance for local and general symptoms in the so-called ' burn syndrome In this context it is of interest that sodium salicylate inhibitor of prostaglandin biosynthesis (Vane 1971) considerably reduces the edema occurring locally after scalding injury (Spector and Willoughby 1959, 1968)

This work was supported by The Swedish Medical Research Council (projects no 13X 2878 13X 217 and 40X-676 the two last mentioned given to Dr B Samuelsson Department of Medical Chemistry Royal Veterinary College Stockholm Sweden and to Dr G Arturson Department of Plastic Surgery University Hospital Uppsala Sweden respectively) The skilful technical assistance of Mrs M Eriksson Mrs. V Lagerstrom and Miss K Wahlberg is gratefully acknowledged

References

ÄNGGÅRD E K GREEN and B SAMUELSSON Synthesis of tritium labelled prostaglandin E_2 and studies on its metabolism in guinea pig lung *J biol Chem* 1965 *240* 1932—1940
ÄNGGÅRD E and C E JONSSON Efflux of prostaglandins in lymph from scalded tissue *Acta physiol scand* 1971 *81* 440—447
ÄNGGÅRD E and B SAMUELSSON Metabolism of prostaglandin E_1 in guinea pig lung the structures of two metabolites *J biol Chem* 1964 *239* 4097—4102
BIRKE C H DUNER S O LILJEDAHL B PERNOW L O PLANTIN and L TROELL Histamine catecholamines and adrenocortical steroids in burns *Acta chir scand* 1957 *114* 87—98
BRUNDIN J The effect of prostaglandin E_1 on the response of the rabbit oviduct to hypogastric nerve stimulation *Acta physiol scand* 1968 *73* 54—57
BYGDEMAN M and B SAMUELSSON Analyses of prostaglandins in human semen *Clin chim Acta* 1966 *13* 465—474
DOUGLAS L G and C E JONSSON Oxygen consumption in skin of experimentally burned rats *Scand J Plast Reconstr Surg* 1969 *3* 23—26
EDERY H and G P LEWIS Kinin forming activity and histamine in lymph after tissue injury *J Physiol (Lond)* 1963 *169* 568—583
GRANSTROM E U INGER and B SAMUELSSON The structure of a urinary metabolite of prostaglandin $F_{2\alpha}$ in the rat *J biol Chem* 1965 *240* 457—461
GREEN K and B SAMUELSSON Thin layer chromatography of prostaglandins *J Lipid Res* 1964 *5* 117—120
GREEN K and B SAMUELSSON Quantitative studies on synthesis in vivo of prostaglandins in the rat Cold stress induced stimulation of synthesis *Europ J bio hem* 1971 *22* 391—395
HAMBERG M and L ISRAELSSON Metabolism of prostaglandin E_2 in guinea pig liver I Identification of seven metabolites *J biol Chem* 1970 *245* 5107—5114
HAMBERG M and B SAMUELSSON The structure of a urinary metabolite of prostaglandin E_2 in the guinea pig *Biochem Biophys Res Commun* 1969 *34* 22—27
HAMBERG M and B SAMUELSSON Metabolism of prostaglandin F_2 in guinea pig liver *J biol Chem* 1971 *246* 1073—1077
HAMBERG M and B SAMUELSSON On the metabolism of prostaglandins E_1 and E_2 in the guinea pig *J biol Chem* In press
HEDQVIST P Studies on the effect of prostaglandins E_1 and E_2 on the sympathetic neuromuscular transmission in some animals *Acta physiol scand* 1970 Suppl 345
JONSSON C E Smooth muscle stimulating lipids in peripheral lymph after experimental burn injury *Scand J Plast Reconstr Surg* 1971 *5* 1—5

JONSSON C E and E ÄNGGÅRD Biosynthesis and metabolism of prostaglandin E_2 in human skin *Scand J clin Lab Invest* 1972 *29* 289—296

JOUVENAZ G H D H NUGTEREN R K BEERTHUIS and D A van DORP A sensitive method for the determination of prostaglandins by gas chromatography with electron capture detection *Biochim Biophys Acta* (Amst) 1970 *202* 231—234

KARIM S M M and K HILLIER A sensitive method for assay of prostaglandins E_1 E_2 F_1 and $F_{2\alpha}$ *Europ J Pharmacol* 1968 *4* 205—210

PACE ASCIAK C K MOROWSKA F COCEANI and L S WOLFE The biosynthesis of prostaglandins E_2 and F in homogenates of the rat stomach In *Prostaglandin symposium of the Worcester Foundation for Exp Biol* Ed P W Ramwell and J E Shaw 1968 Interscience New York 371—378

RAMWELL P W and J E SHAW Spontaneous and evoked release of prostaglandins from cerebral cortex of anesthetized cats *Amer J Physiol* 1966 *211* 125—134

SAMUELSSON B Isolation and identification of prostaglandins from human seminal plasma *J biol Chem* 1963 *238* 3229—3234

SPECTOR W G and D A WILLOUGHBY Experimental suppression of the acute inflammatory changes of thermal injury *J Path Bact* *1959* *78* *121—132*

SPECTOR W G and D A WILLOUGHBY *The Pharmacology of Inflammation* The English University Press London 1968 66—77

UNGER W G I F STAMFORD and A BENNETT Extraction of prostaglandins from human blood *Nature* (Lond) 1971 *233* 336—337

VANE J R Inhibition of prostaglandin synthesis as a mechanism of action for aspirin like drugs *Nature New Biol* (Lond) 1971 *231* 232—235

WEEKS J R J R SCHULTZ and W E BROWN Evaluation of smooth muscle bioassays for prostaglandins E_1 and F *J appl Physiol* 1968 *25* 783—785

ZIBOH V A and S L HSIA Prostaglandin E_2 biosynthesis and effects on glucose and lipid metabolism in rat skin *Arch Biochem Biophys* 1971 *146* 100—109

Acta physiol scand 1973 87 246—251

From the Department of Clinical Physiology Karolinska sjukhuset Stockholm Sweden

Afferent Discharges via the Laryngeal Communicans Related to Respiration in the Rat

By

JAN CASTENFORS EVERT KNUTSSON and TORGNY SJOSTRAND

Received 16 May 1972

Abstract

CASTENFORS, J E KNUTSSON and T SJOSTRAND *Afferent discharges via the laryngeal communicans related to respiration in the rat* Acta physiol scand 1973 87 246—251

Impulse discharges in vagal afferents via the laryngeal communicans and related to respiration have been recorded in Sprague Dawley rats *Particular interest has been devoted to* 2 *types of* discharges one appearing on deflation of the lungs and the other on *active inspiration* The former is *occasionally seen* during ordinary expiration but *especially on application of pressure* on the thorax suction of air through trachea *insufflation of air into the pleural cavity and on* collapse of the lungs by exclusion of artificial breathing with *open* thorax On suction of air and light compression of the thorax impulses *may appear after an initial burst on each expiration.*

The discharges during the inspiratory *phase appear* independently of *inflation of the lungs. They are* very evident during gasps and auxiliary breathing Discharges on *inflation of the* lungs have *only* occasionally been recorded

Afferent discharges via the laryngeal communicans varying with blood volume have previously been described in the rat (Castenfors *et al* 1972) *It was incidentally* reported that discharges appear also *synchronously with respiration* and which increase on hemorrhage Earlier Andrew (1954) *described* respiratory impulses in this nerve but he does not seem to have ascribed any *interest to it* Widdicombe (1954) recorded action potentials in the left *recurrent laryngeal nerve before it reached* the aortic arch and could *identify afferents of three group of tracheobronchial* mechano-receptors but has apparently not recorded impulses via the laryngeal communicans

In the present paper a *more detailed account is presented of impulse discharges in* the laryngeal pathway *apparently related to the respiratory control* The impulses obviously emanate from the airways or lungs *Particular interest has been attached to* two kinds of discharges *one elicited on deflation of the lungs and the other on active inspiration independent of any inflation of the lungs*

Material and Methods

Rats of the Sprague Dawley strain with a b w 250—300 g were used The rats were anesthetized with pentobarbitone (5—8 mg/100 b w) i p The report is based on experiments on more than 60 rats.

As a standard procedure for the nerve recordings the superior laryngeal nerve was exposed between its emergence from the main vagus and entrance into the larynx Its sheath was removed with a pair of thin needles. After lifting and separation of the muscles which cover the laryngeal branches these were cut and the communicating branch between the superior and inferior laryngeal nerves was laid bare All its connections were then cut under a dissection microscope except for those with the superior and inferior laryngeal nerves Branches of the inferior laryngeal nerve to the trachea were also severed over a distance of about 1 cm The superior laryngeal nerve was then cut off at its entrance into the vagus The nerve was placed on a couple of Ag AgCl electrodes The nerve could then be kept hanging in the air at a distance of about 1 cm between the larynx and the recording electrodes which eliminated pick up of electromyographic activity from adjacent muscles

Recordings were made placing the nerve at different sites in relation to the electrodes and thus different types of activity were observed In about half of the recordings the end of the nerve was split into thinner bundles and the discharges from different parts of the nerve were checked

The nerve impulses were amplified in a differential amplifier (Grass P9 or TP3) with linear frequency response between 100 and 10 000 Hz. The signals were fed to one of the inputs of a double beam oscilloscope to a loud speaker and for later display and analysis to a FM tape recorder (Tandberg)

In some cases the taped neurograms were rectified and time averaged (time constant equal to 0 2 s) in a Grass polygraph integrator to facilitate estimation of the changes in total nerve activity

Respiratory movements were indicated by means of one of 2 alternative methods Usually a tube of plastics filled with mercury (inner diameter 1 mm) was attached around the thorax It was connected to a bridge-coupled amplifier and expansion of the thorax was recorded by the resistance changes in the tube In the other method a volumetric pressure transducer (Grass PT 5 A) was connected to the tracheal catheter via an air filled tube Also this transducer was connected to the bridge-coupled amplifier In both cases the dc signals were fed to an ink writer and to one of the beams of the oscilloscope Usually the signals were also recorded on the tape recorder for later display and analysis

Inflation and suction of air were done through the tracheal cannula by means of a syringe To alter the intrathoracic pressure and the movements of the lungs pressure was applied manually over the thorax Pneumothorax was effected by introducing a catheter connected to a water manometer By gentle movements of the tip after its entrance through the wall of the thorax a satisfactory location of the tip was signalled by the typical intrapleural pressure changes A predetermined amount of air was then introduced via the catheter from a syringe connected to the manometer Respiratory movements in these experiments were recorded with the volumetric pressure transducer which indicated change of level as well as of amplitude of the recorded respiratory movements on insufflation of air into the intrapleural space Artificial respiration was applied by rhythmical interruptions of air or oxygen from the general pressure supply Volume and frequency could be adjusted

Results

Different discharges from the laryngeal pathway have been observed some described in a previous paper (Castenfors *et al* 1972) In the present paper only discharges associated with respiration are reported These are all elicited mechanically We have never observed impulse discharges which could be referred to chemical stimulation as on an increase of the dead space by spontaneous breathing through a tube or after exclusion of the artificial respiration with the thorax open

Discharges in eupnea

Expiratory discharges during spontaneous breathing have been observed in about

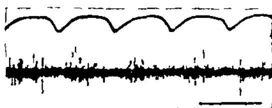


Fig 1 Expiratory discharges from the laryngeal pathway during spontaneous breathing in the rat The recording was made from the left superior laryngeal nerve after cutting proximal to the recording site and severance of all its branches to the larynx The upper curve shows the respiratory movements recorded with a strain gauge transducer cable around the thorax Downward deflexions indicate inspirations The lower curve is the recording of nerve impulses Time bar 0 5 s

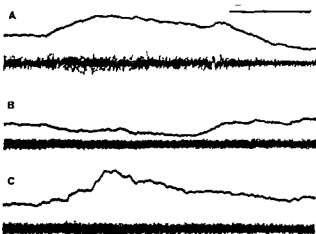


Fig 2 Recording of the responses to withdrawal of air through the tracheal cannula (A) manual pressure applied over the liver (B) and over the thorax (C) The lower curves are the neurograms recorded from the left superior laryngeal nerve after dissection as in the experiment of Fig 1 The upper curves are integrated neurograms to indicate alterations *in the total nerve activity* Dotted lines indicate duration of stimuli and the horizontal bar in A is 0 5 s

10 % of the recordings (Fig 1) Discharges during the inspiratory phase have also been recorded especially after drawing off blood (see Castenfors *et al* 1972 Fig 2) We shall return to these subsequently

Discharges provoked by deflation of the lungs

Lung deflation was provoked by different means such as applying manual pressure *to the thorax* sucking out air *from the trachea insufflation of air into the* pleural cavity exclusion of the artificial respiration with open thorax Under all these conditions the discharges increased as exemplified in Fig 2—6 On maintained stimulation the discharges are of rather short duration and decrease in frequency During the deflation of the lungs with the thorax open the bursts appear when the deflation has reached a certain degree seemingly independent of the initial lung volume

On a decrease of the thoracic volume by application of a light pressure to the thorax as well as on sucking of air from the trachea expiratory discharges may appear, as seen in Fig 3 and 4 The duration of the discharges varies with the expiratory phase Continuous activity on compression of the thorax or on pneumothorax has not been observed *In preparations with a tonic activity* (see Castenfors *et al* 1972) a pressure applied to the abdomen usually decreases the activity (Fig 2 B)



Fig 3



Fig 4

Fig 3 Neurogram from the left superior nerve (lower curve) in a preparation without any spontaneous activity but showing bursts of impulses during expiratory movements in response to pressure applied to the thorax after the first respiratory cycle The upper curve indicates respiratory movements a downward deflexion depicting inspiration Time bar 1 s

Fig 4 Recording from the superior laryngeal nerve (upper curve) after cutting both vagi as well as the branches to the larynx In this preparation there was a response to withdrawal of air via the tracheal cannula (after the first two respiratory cycles) appearing as a burst of impulses during expiration Respiratory movements shown by lower curve downward deflexion indicates expiration Record disturbed by ECG Time bar 0 5 s

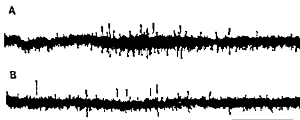


Fig 5 Impulse discharges transmitted via the laryngeal communicans in response to deflation of the lungs Thorax open and deflation indicated by upper horizontal bars in the consecutive recordings in A and B Time bar in B 0 5 s

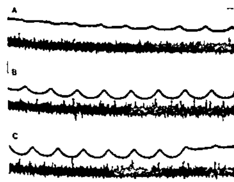


Fig 6

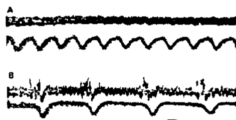


Fig 7

Fig 6 The alterations of impulse discharge in the laryngeal pathway during insufflation of air into the intrapleural space Insufflation starts in A and continues in the consecutive recordings in B It is indicated in the recording of respiratory movements (upper curves) by the successive increase in amplitude of movements and change of level A piration of air at the end of record C Upward deflexions indicate inspiration Time bar in C 1 s

Fig 7 Illustrates the arousal of inspiratory discharges in the laryngeal pathway after withdrawal of blood In A control without any spontaneous activity The upper curve is the neurogram and the lower curve depicts respiratory movements downward deflexions indicate inspirations In B recording after withdrawal of 3 ml of blood via a femoral catheter In this record artefacts by ECG and by electrode displacement on inspiratory movements Time bar in B 1 s

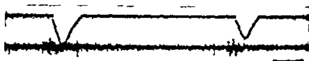


Fig 8 Inspiratory bursts of impulses from the laryngeal pathway Thorax open and artificial respiration stopped Upper curve strain gauge records of changes of the thoracic cage inspiration downward Lower curve neurogram Time bar 0 5 s

Inspiratory discharges

Bursts of action potentials have been recorded on inspiration, especially on deep breathing gasps and subsequent to hemorrhage (Fig 7) However only in 1 expt have discharges been observed on passive inflation of the lungs In the remaining observations the activity has appeared on active inspiration apparently independent of any inflation Under artificial respiration with open thorax this has been evident Thus on exclusion of the respiration a marked activity on spontaneous inspiratory movements of the opened thorax has appeared without any change of the lung volume Fig 8

A close analysis of the activity has revealed that it starts before the inflation of the lungs and lasts only during the initial part of the inspiration thus disappearing before the maximum of an eventual inflation (Fig 7) The activity remains after cutting the vagus trunk bilaterally

Discussion

Discharges on deflation

Afferent discharges from the lungs on deflation have been described by several uthors since Keller and Loeser (1929) and Adrian (1933) made the first observations but the specificity of the allied receptors has been questioned also their function The existence of a deflation reflex is also well established but it does not seem to play a part in the self regulation of respiration (Koller and Ferrer 1970) Thus these authors state that the role of the deflation reflex is to prevent and supress pulmonary compression and collaps and the reflex is excited by any decrease of lung volume below the normal relaxation volume The probable receptors allied to the deflation reflex have recently been reviewed and pulmonary stretch receptors lung irritant receptors and the J receptors earlier named specific deflation receptors by Paintal (1955) have all been ascribed a role under different conditions (Fillenz and Widdicombe 1972)

However the results of the present study indicate that there are receptors in the rat which are stimulated on deflation but not by inflation of the lungs and which may be activated during ordinary expiration Accordingly it seems less probable that the specific pulmonary stretch receptors are the receptors in question It seems not probable either that the lung irritant receptors are activated on spontaneous expiration The J receptors are sometimes stimulated by deflation of the lungs and by

pneumothorax but they have been removed from the list of supposed specific de flation receptors because their response to deflation is only transient and weak

Receptors in the vascular system may play some part at least on compression of the thorax and on pneumothorax. Thus a coincident decrease of the central blood volume would stimulate the LL receptors which also discharge via the laryngeal pathway (Castenfors *et al* 1972) The decreased tonic impulse activity on applica tion of pressure below the diaphragm may be explained by an increased inflow of blood to the thorax and a subsequent decreased stimulation of the LL receptors However suction of air via the trachea should have the same circulatory effect but elicits discharges in the same preparation The variations with respiration also tell against a vascular origin of the discharges

At present we refrain from any speculations on the nature and function of the receptors underlying the deflation discharges and only refer to our observations which seem to indicate that there are receptors which may be stimulated on ordinary expiration especially when the thoracic mean volume is lowered and which ap parently have not been described earlier

Inspiratory discharges

The activity preceding active inflation of the lungs would seem most likely to be elicited from innervated structures taking part in the respiration We assume that the allied receptors are localized to the musculature in the airways However the activity remains after cutting the vagus trunk

Contrary to the vagal trunk the laryngeal communicans seems only occasionally to convey discharges from pulmonary stretch receptors This indicates that there is a systematic distribution of afferents from the lungs between the laryngeal path and the vagus trunk In the same way as has been done in respect of the circulatory con trol this may be utilized for further studies on the pulmonary receptor control mechanisms

References

ADRIAN E D Afferents impulses in the agus and their effect on resp ration *J Physiol* (Lond) 1933 *79* 332—358

CASTENFORS J E KNUT ON and T SJOSTRAND C rculatory control via vagal afferents II Effects of hemorrhage on afferent discha ges ia the laryngeal commun an in the rat *Acta phys ol scand* 1972 *84* 355—365

FILLENZ M and J G WIDDICOMBE Receptors of the lungs and airways In Enteroceptors Ed E Neil Springe Verlag Berl n He delberg New York 197?

KELLER C J and A LOESER Der entrip tale Lungen agus *Z B I* 1929 *89* 3 3—395

KOLLER E A and P FERRER Studies on th role of the lung deflation reflex *Resp Phy iol* 1970 *10* 172—183

PAINTAL A S Imp lses in vagal afferent fib es from pec f c pulmonary deflation receptors The respon of the rec ptors to phen l diguan de potato tarch 5-hydroxytryptamine and nicotine and th r rol in espiratory and card o ascular reflexes *Q t J exp Physiol* 1955 *40* 89—111

WIDDICOMBE J G Receptors in th trachea and b onchi of the cat *J Phys l* (Lond) 1954 *123* 71—104

Acta physiol scand 1973 87 252—260

From the Institute of Medical Physiology Dept A University of Copenhagen Denmark

Comparative Studies of Capillary Permeability in Brain and Muscle

By

CHRISTIAN CRONE and ALAN M THOMPSON*

Received 20 May 1972

Abstract

CRONE C and A M THOMPSON *Comparative studies of capillary permeability in brain and muscle* Acta physiol scand 1973 87 252—260

The permeability of the capillary wall in the brain was compared with that in muscle by means of the ratio between outflow concentrations (relative to inflow concentration) after single i a injections of various combinations of two radioactive tracers A ratio above 1 means that the solute in the denominator left the capillaries faster than the other tracer *vice versa* The following outflow ratios were obtained *Hind limb* Glucose/K and 3 O methyl glucose/K above 1 *Brain* Glucose/K and 3 O methyl glucose/K below 1 The hind limb results show a faster transcapillary transport of potassium than of the sugars—in agreement with the predictions of the Pappenheimer pore model for muscle capillaries In brain the sugars are transported faster across the capillary wall than tracer potassium This finding cannot be explained by diffusion through a common interendothelial diffusion pathway (pore) but signify that the sugars are transported through the capillary endothelial cells in brain—a route not available to potassium The Pappenheimer pore model only applies to the brain capillaries as a limiting case with a pore permeability close to zero even for small solutes *Thus the permeability* characteristics of the brain capillaries are in closer agreement with those of monolayered epithelia than with the Pappenheimer pore model It is concluded that important blood brain barrier functions are carried out in the cerebral capillary walls

The most useful model of the capillary membrane is the one worked out by Pappenheimer (1953) who described it as being equivalent to a multi porous passive membrane displaying diffusion restriction towards low molecular weight solutes Although the morphological equivalent to capillary pores is not known for certain the interendothelial cleft is considered the most likely candidate Muscle capillaries have interendothelial passages where the narrowest width is about 40 Å (Karnovsky 1967) Brain capillaries on the other hand have circumferential tight junctions between neighbouring endothelial cells (Brightman and Reese 1969) The dimension of the narrowest width in a tight junction is not definitely known but it is of course much smaller than 40 Å This means that diffusion restriction must play a very prominent role for the well known impedance to exchange of solutes between blood and brain tissue If the narrowest width in a brain capillary pore is about 15 Å the diffusion of a molecule like glucose is severely restricted

* On leave of absence from Dept of Physiology Univ of Kansas Medical Center Kansas City USA

In order to contrast the behaviour of two capillary beds with very different dimensions of the interendothelial pore experiments were performed with several low molecular hydrophilic test solutes The single injection method (Crone 1963) was chosen as experimental method because it gives information about the permability of the capillary membrane as reflected in the extraction of tracers after a single passage through the organ

Another way of demonstrating permeability differences between muscle and brain capillaries consists in calculating ratio of the concentration s of 2 test solutes in the effluent blood after a single passage of a mixture of 2 tracers

The experiments showed that brain capillaries are much less permeable than muscle capillaries but the main finding was that there are not only quantitative differences between muscle and brain capillaries The passage of D glucose from blood into brain cannot solely be explained as diffusion in narrow pores but must include passage through the endothelial cells a feature which is not displayed by muscle capillaries

Methods

The experiments were performed on mongrel dogs anesthetized with sodium pentobarbital (nembutal) 25—30 mg i v A free air passage was ensured by means of an endotracheal tube The animals were pontaneously breathing

For the *brain* experiments a small polythene cannula was introduced through the superior thyroid artery almost into the lumen of the common carotid artery so that injections could be given without interference with the blood flow in the carotid artery A craniotomy just above the superior sagittal sinus immediately in front of the torcula exposed the sagittal sinus. A Perpex adapter (Rodnight and Tresize 1957) was screwed firmly into the scull and a polythene catheter was attached to this device At the time of the experiment an incision was made in the sinus so that the blood could flow f eely through the sampling system Small collecting vials containing dry heparin were mounted on a slo ly moving chymograph to collect the effluent cerebral blood fractionally The total sampling time was about 20 s

Approximately 1 ml of a test solut on was injected quickly and sampling was begun immediately fterwards In all cases the injection solution contained 2 different test solutes

For the *muscle* experiments the femoral artery and vein were dissceted free in the inguinal region A mall side branch of the femoral artery was hosen for injection and a polythene tube was introduced into the vessel Sampling took place via a polythene tube inserted into a side branch of the femoral vein With this arrangement injection and sampling could take place without any flow interference The femoral and sciat c nerves were solated and cut Stimulating electrodes were placed in contact with the cut ends The muscles were stimulated with rectangular pul es from a G ass stimulato with a frequency of 1/s The experiments were performed on contracting muscles in order to increase blood flow so that flow l mitat on would not play a significant role During the exper ments a firm l gature was placed around the ankle to prevent distribut on to the paw of the injected solution Sampl ng took place as described for the brain experiments

All solutes under n st gat on were u ed n labell d form ^{14}C D glucose ^{14}C 3 O methyl glucose T mannitol ^{4} K and ^{2} Na The radioacti e compounds were del vered from The Danish Atomic Energy Commission from The Radiochemical Centre Amersham and from New England Nuclea Corporation

Determination of sotope concent ations of blood samples took place in eith r a Packard Liquid Scintillation Spectrometer (model 3003) or in a Packard Automatic Gamma Spectrometer (model 3003) In experiments with ^{4} K and some other isotope advantage was taken of the ho t half time of ^{4} K and ountings were repeated after a week for determination of the other isotope present Tritiated mannitol and ^{14}C labeled glucose were determined simultaneously using 2 channels

The blood samples were p ecipitat d with 5 trichlo o-acetic ac d and 0 3 ml of the supernatant was added to 15 ml scintillat on mixture for counting of β emission The γ activity was determined on the entire volume of precip tate and supernatant

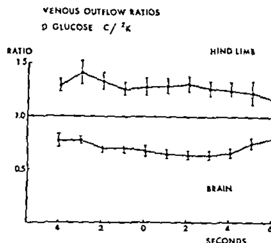


Fig 1

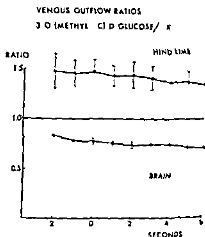


Fig 2

Fig 1 Venous outflow concentration ratios of ^{14}C D glucose and ^{42}K in experiments on brain and hind limb Abscissae Time in seconds the peak of the dilution curve is arbitrarily called zero-time Ordinates Concentration ratios the vertical bars indicate ± 1 SE

Fig 2 Venous outflow concentration ratios of 3 O ^{14}C-methyl glucose and ^{42}K in experiments on brain and hind limb Abscissae and ordinates as in fig 1

The experimental and analytical techniques have been in regular use in the laboratory for several years and additional information is found in Crone (1965)

Expression of results All concentrations in the effluent blood were expressed relative to those in the injection mixture (relative concentration) As most of the experiments aimed at determining which of either isotope in the mixture left the capillaries more rapidly 2 isotopes were always injected at the same time The results were expressed as the ratio between the relative concentrations of the 2 isotopes in the effluent blood If the ratio was above 1 the isotope in the denominator of the ratio had left the circulation more rapidly than the other *vice versa* The method was judged valuable for determining which of two substances diffused through the capillary wall fastest

Other experiments aimed directly at determining the extracted fraction of the injected isotope during one passage through the brain In these experiments inulin was used as reference substance because this solute does not leave brain capillaries to any significant extent during a single passage

Results

1 ^{14}C D glucose/^{42} potassium outflow ratios

The main question was How does D glucose get through brain capillaries? To evaluate this a mixture of ^{14}C D glucose and ^{42}K was injected i a into brain and into a hind limb The concentration ratios of the 2 substances were determined in the outflowing blood Altogether 5 expts were performed on brain and muscle respectively It was found in all instances that the ^{14}C D glucose/^{42}K ratio in brain was below 1 while in muscle it was above 1 The results are shown on Fig 1 The finding of a ^{14}C-D glucose/^{42}K ratio above 1 reflects a faster diffusion of potassium than of D glucose out of muscle capillaries— a completely expected result However these results show unequivocally that D glucose leaves the brain capillaries

TABLE I Mean ratios of relative outflow concentrations after injection in supplying artery

Dog brain		
K/T mannitol	1 019 ± 0 010 (SE)	6 experiments n = 56
C-D glucose/T mannitol	0 685 ± 0 058	4 experiments n = 41
K/ Na	0 956 ± 0 005	6 experiments n = 48
Dog hind limb		
K/T mannitol	0 842 ± 0 008 (SE)	10 samples
	0 716 ± 0 017	23 samples
C-D glucose/T mannitol	0 945 ± 0 003	9 samples
	1 001 ± 0 004	11 samples

* Means of all samples SE for the number of samples indicated
* Means of single experiments SE for the number of samples indicated

faster than does potassium a result which is not consistent with the ordinary capillary pore model It might be argued that because the brain consumes glucose at high rate the concentration is kept low outside the capillary while the labelled potassium piles up in the interstitial space Back flux of labelled potassium might thus lead to an underestimation of the true extraction of this ion Similar experiments were therefore carried out with labelled 3 O methyl glucose this compound not being metabolized

2 3 O ^{11}C methyl D glucose/^{42}K ratios

The experiments with the non metabolized glucose analog gave nearly similar results as seen from Fig 2

The experiments thus demonstrate that the faster transport of D glucose out of brain capillaries is not due to it being metabolized and the conclusion is that a transport mechanism for D glucose must be located in the capillary membrane itself

3 ^{11}C D glucose/T mannitol ratios

Glucose and mannitol were chosen for comparative experiments on brain and muscle because of the great physical similarity of the two molecules and because their free diffusion coefficients are nearly the same

The results are given in Table I from which it is seen that in the brain the outflow ratio between D glucose and mannitol was below 1 while in the hind limb the ratio was very close to 1 The finding of a ratio in the hind limb of 1 is to be expected from the usual capillary model while the brain results certainly suggest some additional mechanism for glucose transport The experimental findings thus are in accordance with the results of the glucose K experiments

4 ^{42}K/T mannitol ratios

Potassium having a higher diffusion coefficient than mannitol would under all circumstances diffuse faster through a passive physical membrane with uncharged pores This was the case in experiments on the hind limb as reflected in a ^{42}K/T-mannitol ratio below 1 (Table I)

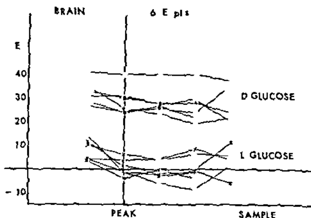


Fig 3 Initial extraction of labelled D and L-glucose in 6 Indicator Diffusion experiments on brain The two tracers were injected simultaneously The extraction was calculated relative to inulin Ordinates Extracted fraction of either test solute (E)

In the brain however the ratio between the two was essentially 1 (Table I) This implies that virtually none of the 2 test solutes have left the system during one passage through the brain The experiments thus demonstrate an extreme tightness of brain capillaries as compared with muscle capillaries

5 $^{42}K/^{22}Na$ *ratios in brain effluent blood*

It is not known whether there are differences between the rate of transport of potassium and sodium ions through brain capillaries Determination of the concentration ratios in the venous effluent is a sensitive way of assessing this problem The transport of the 2 ions is in such experiments studied under conditions which are similar for all variable parameters The ratio $^{42}K/^{22}Na$ was 0 988 not significantly different from 1 (Table I) The experiments thus show that differences in transcapillary transport rates in brain are not measurable with this method

Similar experiments have been performed on muscle by Yudilevich *et al* (1968) who found that ^{42}K leaves capillaries faster than does ^{22}Na

6 *Extractions of D and L glucose*

So far stereo-specific mechanisms have not been demonstrated in transcapillary transport although the above postulated presence of facilitated transport through brain endothelium certainly would imply the existence of a stereo-specific transport mechanism

6 expts were performed in which tritium labelled D glucose and ^{14}C labelled L glucose were injected together with inulin as reference substance It was assumed that sucrose stayed intravascularly during the capillary transit and the extraction of D and L glucose respectively was calculated for each sample obtained before and after the peak of the dilution curve Fig 3 shows the pronounced difference between the extractions of the two optical isomers studied under conditions which were similar for the two substances as regards flow capillary surface area etc. Fig 4 shows a typical time-concentration curve

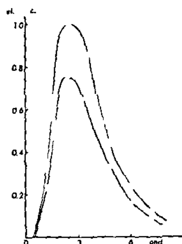


Fig 4 Typical venous outflow time-concentration curves for inulin (circles) and Labelled D-glucose (crosses) Injection into carotid artery at 0-time. The curves are normalized so that the highest relative concentration is 1 L-glucose was injected in the same experiment but it fell so close to the inulin curve that it was omitted on the sketch

The remarkable difference between the extraction of the two substances is obvious from the very first sample as seen from the figure The fact that the difference occurs so early speaks against the explanation being due to piling up of the non metabolizable L-glucose in a small space just outside the capillary

A separate study of the transcapillary transport of D and L-glucose in perfused muscle (Crone 1972) showed a slightly greater extraction of D glucose than of L glucose but nothing comparable to what was found in the present experiments on brain

Discussion

The reason for making comparative experiments on muscle and brain capillaries was that structural and functional characteristics of muscle capillaries are quite well known Any differences in the behaviour of test solutes in the 2 systems of capillaries might therefore disclose special features on the brain capillaries

The experiments have amply demonstrated permeability differences between muscle and brain capillaries and the brain results are not easily interpreted according to the general Pappenheimer capillary model (Pappenheimer 1953) This model which was worked out for muscle capillaries states that the transcapillary transport takes place via the pores or slits between endothelial cells Due to the fact that the dimensions of these diffusion paths are down to almost molecular dimensions the phenomenon of restricted diffusion is observed This phenomenon being present in muscle capillaries with pore diameters of about 40 Å at the narrowest point must by necessity be much more pronounced in cerebral capillaries where the pores are so narrow that they appear as tight junctions in electronmicroscopic pictures (Brightman and Reese 1969) In fact it is somewhat of a problem to explain how solutes at all pass a capillary with such a tight endothelium

The results of the experiments with ^{42}K and labelled mannitol showed that in brain the outflow ratio was about 1 which fits well with the fact that hardly any of the solutes leave the plasma during a passage through the brain In contrast similar experiments on muscle gave the expected result namely a ^{42}K/T mannitol ratio below 1 explained by a slower mannitol than potassium diffusion in the interendothelial pore

The brain experiments with ^{42}K and ^{22}Na gave an outflow ratio which was very close to 1 a finding which again suggests that hardly any of the two ions left the circulation during the passage through the brain This is in contrast to what has been found in similar experiments on muscle capillaries where ^{42}K escapes faster than Na (Yudilevich et al 1968) but in complete agreement with the experiments of Lassen et al (1971) on brain capillaries

The present experiments thus once more demonstrate the extraordinary tightness of the cerebral capillary wall

All the experiments in which D glucose was studied showed that this solute traversed the brain capillary wall faster than any other solute with which it was compared (potassium mannitol or L glucose)

The experiments with the combination tritiated D glucose and ^{42}K are specially noteworthy because they gave so unequivocal results In muscle capillaries the expected values above 1 of the outflow ratios of D glucose/potassium were invariably found in accordance with a faster transcapillary transport of potassium In brain the opposite was always found This result cannot be explained by applying the Pappenheimer capillary model to the brain According to this model potassium has to leave the circulation faster than D glucose The results are in fact rather striking the more so as similar findings were made with the non metabolizable 3 O methyl glucose (see Fig 1 and 2)

In view of the great tightness of the brain capillary endothelium the results to us signify that D glucose passes through the endothelial cell proper probably by a facilitated diffusion mechanism It is reasonable to postulate a specialized transport mechanism in view of the present demonstration of a clear difference in transport rate of D and L glucose (also found by Oldendorf (1971) and Yudilevich and de Rose (1971) and the earlier finding that the mechanism is saturable (Crone 1965)

Pappenheimer (1970 a and b) has advocated the view that the fast transport of D glucose across the blood brain barrier is explained by a rapid transport through the pericapillary glial foot processes which lie close to the basement membrane of the capillaries Such a trans glial transport cannot be excluded on present evidence but in case it exists it must exist together with a transendothelial glucose transfer

The brain capillary endothelial wall has many of the characteristics of a flat epithelium (e g the thin limb of Henle in the kidney) Epithelia have characteristically tight junctions between individual cells (Farquhar and Palade 1963) just as the brain endothelial cells Tight junctions in epithelia can reversibly open up with hypertonic solutions on the outside (Ussing 1965) A similar osmotic opening

phenomenon has been observed in brain (Thompson 1969) The degree of leakyness of an epithelium seems to depend on the tightness of the tight junctions between epithelial cells (Diamond and Fromter 1972) It appears that a spectrum of leakyness exists between various epithelia Where the brain capillaries belong with respect to leakyness is at the present time difficult to assess with great accuracy due to obvious experimental difficulties encountered in *in vivo* experiments as compared with flux measurements on isolated epithelia. Electric potential differences are usually present across an epithelial structure and it exists across the blood brain barrier (Loeschke 1971)

The resuts presented in this article are very difficult to reconcile with Pappenheimer s proposal (1970 a and b) that the pericapillary glia cells perform the long erm regulation of the composition of the brain interstitial fluid First of all diffusion in the 200 Å spaces between the glia cells is relatively free (Nicholls ana Kuffler 1964) Secondly the demonstrated tightness of the brain capillaries proper together with their ability to facilitate diffusion of certain solutes means that this structure has to be taken into account when discussing the morphological substrate of the blood brain barrier Whether the pericapillary glia cells contribute to defining the blood brain barrier cannot be excluded at the present state but positive evidence that this is the case is seriously lacking

Expert technical assistance from miss Bente Mertz is gratefully acknowledged Economical support was received from The Danish Heart Association The results of the D and L-glucose experiments v e e communicated at the Second Alfred Benzon Symposium Copenhagen 1969

References

BRIGHTMAN M W and T S REESE Junctions between ultimately apposed cell membranes in the vertebrate brain *J cell B ol* 1969 *40* 648—677

CRONE C The permeability of capillaries in various organs as determined by use of the Indicator Diffus on method *Acta phys ol scand* 1963 *58* 292—305

CRONE C The permeability of brain capillaries to non electrolytes *Acta phys ol scand* 1965 *64* 407—417

CRONE C Facilitated transfer of glucose from blood into brain tissue *J Phys ol* (Lond) 1965 *181* 103—113

CRONE C D and L-glucose permeabil ty in muscle capillarie *Acta physiol sca d* 19 2 In press

DIAMOND J and E FROMTER Route of passi e ion permeation n epithelia *Nature* (Lond) 197? *235* 9—13

FARQUHAR M G and C E PALADE Junctional complexes n various epithel a *J cell Biol* 1963 *17* 375—412

KARNOVSKY M J The ultrastru ture bas s of capillary permeability stud ed with p rox dase as a tracer *J cell B ol* 1967 *35* 213—236

LASSEN N A J TRAP JENSEN S C ALEXANDER J OLESEN and O B PAULSON Blood brain barrier studie in man u ng the double indicator method *Ame J Physiol* 1971 *220* 1627—1633

LOESCHKE H H DC potent al between CSF and blood In *Ion hor e stasis of the b ain* Ed t B K Siesjo and S C So nsen Munksgaard 1971

NICHOLLS J E and S W KUFFLER Extracellular space as a pathway fo exchange between blood and neurons n th central n rvou sy tem of the leech Ion c composition of glial cell and neurons *J Neurophy ol* 1964 *27* 645—671

OLDENDORF W H Brain uptake of radiolabelled amino ac ds amines and hexoses after arterial injection *A er J Phy iol* 1971 *221* 1629—1633

PAPPENHEIMER J R Pas age of molecules through capillary walls *Phys ol R v* 1953 *33* 387—423

PAPPENHEIMER J R Transport of HCO_3 between brain and blood In Alfred Benzon Symposium II *Capillary Permeability* Edit C Crone and N A Lassen pp 451—458 1970 a Munksgaard Copenhagen

PAPPENHEIMER J R On the location of the blood brain barrier In *Proc of a Symposium on the blood brain barrier* Truex Press Oxford Edit R N Coxon 1970 b

RODNIGHT R and M A TRESIZE A method for obtaining simultaneous samples of venous blood from the brain in conscious rabbits *J Physiol* (Lond) 1957 *136* 71P

THOMPSON A M Hyperosmotic effects on brain uptake of non electrolytes In Alfred Benzon Symposium II *Capillary Permeability* Edit C Crone and N A Lassen pp 459—461 1970 Academic Press New York

USSING H H Relationship between osmotic reactions and active sodium transport in the frog skin epithelium *Acta physiol scand* 1965 *63* 141—155

YUDILEVICH D L E M RENKIN O A ALVAREZ and I BRAVO Fractional extraction and transcapillary exchange during continuous and instantaneous tracer administration *Circulat Res* 1968 *23* 325—336

YUDILEVICH D L and N DE ROSE Blood brain transfer of glucose and other molecules measured by rapid indicator dilution *Amer J Physiol* 1971 *220* 841—846

Acta physiol scand 1973 87 261—269

From the Institute of Neurophysiology University of Copenhagen and the Laboratory of Clinical Neurophysiology University Hospital (Rigshospital) Copenhagen Denmark

Rise Time of the Spike Potential in Fast and Slowly Contracting Muscle of Man

By

FRITZ BUCHTHAL KRISTIAN DAHL and POUL ROSENFALCK

Received 30 May 1972

Abstract

BUCHTHAL F K DAHL and P ROSENFALCK *Rise time of the spike potential in fast and slowly contracting muscle of man* Acta physiol scand 1973 *87* 261—269

In 20 muscles of 10 normal subjects rise times of the spikes of motor unit potentials were recorded during weak voluntary effort by means of a concentric electrode with a small tip area (0 015 mm²) Rise times in the soleus muscle which consists mainly of slowly contracting fibres were twice as long on the average as in the lateral head of the brachial triceps which *contains mainly fast contracting fibres* $(190 \pm 10\ \mu s$ *145 spike potentials compared to* 107 ± 4 μs 115 spike potentials) In both muscles the distribution of rise times was skewed toward the long rise times The longer rise times in the soleus muscle could not be accounted for by smaller diameter of the muscle fibres and therefore slower conduction velocity than in the lateral head of the brachial triceps Therefore the properties of the excitable membrane must be different in the slow fibres of the soleus from those of the fast fibres of the lateral head of the brachial triceps The difference in rise time of the spikes in the two muscles was not reflected in the total duration of the motor unit potentials recorded by means of conventional concentric electrodes

The fibres of skeletal muscle differ with respect to speed of contraction enzyme content and number of mitochondria (*cf* Henneman and Olson 1965) Long contraction times predominate in those muscles whose fibres are rich in mitochondria (*e g m soleus*) and short contraction times in muscles which are poor in mitochondria (*e g* m triceps brachii caput laterale) (Buchthal and Schmalbruch 1970)

In the cat s soleus and gastrocnemius muscle small slowly contracting motor units were innervated by slowly conducting axons large fast motor units by fast conducting axons (McPhedran Wuerker and Henneman 1965 Wuerker McPhedran and Henneman 1965) This relation was however not confirmed either in different muscles or within the faster contracting population of a single muscle (Stuart Goslow and Gerlach 1971)

With the inaccurate methods available then Babkin (1908) found a different time course of the action potential in fast and slowly contracting muscles of frog and Kohlrausch (1912) described that the wave of negativity in the slow soleus mus

of rabbit and cat was transmitted at a slower rate than in the faster gastrocnemius. Since then there have been studies of the properties of the excitable membrane of muscle fibres with different speed of contraction. In cat Eccles and O'Connor (1939) found that the action potential was conducted more slowly along the slow soleus muscle than along the faster peroneus tertius and anterior tibial muscles. Similarly, by intracellular recording the conduction velocity and rise time were 20—25 % faster in fibres of the fast flexor hallucis longus than in the slow soleus muscle of cat (Buller, Lewis and Ridge 1965). As to the resting potential findings differed in muscles of cat and rat. Buller, Lewis and Ridge (cited in Lewis 1972) found a higher membrane potential in fast than in slow muscles of the cat whereas Lorkovic (1971) found no difference either in the membrane potential or the threshold potential for mechanical activity in fast and slow muscles of the rat.

The purpose of the study presented here was to investigate the excitable membrane of human muscle fibre. To this end the rise time of the spike of the motor unit potentials was recorded in two muscles in humans, in the soleus muscle which contains only slowly contracting fibres, and in the lateral head of the brachial triceps which contains mainly fast contracting fibres. If there were a difference, was it related to the size of the muscle fibre and could it be detected in the compound potential of the motor unit?

Method

1. Recording of the spikes of the motor unit potentials

Electrodes. To record the rise time of the spikes, concentric electrodes with a 0.015 mm² tip area of the platinum core were used. The outer diameter of the cannula was 0.3 mm. To diminish the noise, the impedance of the platinum core was reduced by passing through it an a.c. current of 10 mA for about 5 s when the electrode was immersed in saline at 90 °C. When an air bubble had formed at the tip the total impedance in 0.15 NaCl was 100 kΩ at 300 Hz and 200 kΩ at 35 Hz, about 10 times lower than before passage of current.

To record the total duration and amplitude of the motor unit potential concentric electrodes with a 0.07 mm² tip area were used. The outer diameter of the cannula was 0.65 mm. The total impedance was 20 kΩ at 300 Hz and 100 kΩ at 35 Hz.

Amplifier and delay line. The spike potentials were recorded at a gain of 0.25—0.5 mV/cm. The a.c. amplifier (DISA 14C11) had an upper limiting frequency of 10 000 Hz (3 db down) and an input impedance of 250 MΩ shunted by 15 pF (balanced). The compound motor unit potentials were recorded at a gain of 0.1 mV/cm when the total duration, and at 0.3 mV/cm when the peak-to-peak amplitude was to be measured.

To superimpose 5—10 spike potentials photographically, the spikes triggered the sweep of the cathode ray oscilloscope (Tektronix 564) and were passed through a delay line (DISA 14B80) with a delay of 1 ms (Nissen-Petersen, Guld and Buchthal 1969). The upper limiting frequency transmitted by the delay line was 10 000 Hz (3 db down) and the cut-off frequency was 12 000 Hz (40 db). Recording the same spike potential with and without the 1 ms delay showed that the delay did not affect the rise time even when there was an oscillatory response ("ringing") with rise times of less than 100 μs (Fig. 1). The time base was 100 μs/division, one division corresponding to 10 mm on the photograph used for measurement. To ascertain that the spike potentials superimposed on the oscilloscope screen originated from the same motor unit potential, the motor unit potentials were recorded simultaneously on single sweeps on the oscilloscope of the electromyograph (DISA 14A30) with a total sweep duration of 7.5 ms. To display the whole duration of motor unit potentials the delay was 10 ms and the duration of the sweeps was 30 ms.

The *intramuscular temperature* was kept near 36 °C by heating the extremity and by controlling the heat supply automatically. An infrared heating element 45 cm long (200 W) was placed 20 cm above the extremity. It was controlled by a thermocouple on the skin of the arm or leg. The intramuscular temperature was measured by means of a thermocouple at the tip of a cannula inserted near and to the same depth as the recording electrode.

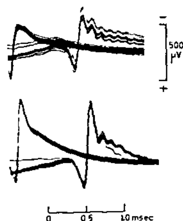


Fig 1 Spikes of motor unit potentials recorded directly (left) and via a delay line of 1 ms (right) Even the shortest rise times (above 60 µs below 75 µs) remained unaltered The decline of the spike was distorted in the delay line by "ringing"

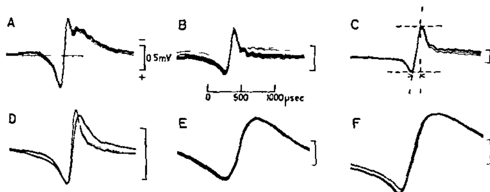


Fig 2 Typical examples of spikes of motor unit potentials recorded in the lateral head of the brachial triceps (A, B C) and in the soleus muscle (D E F) of the same subjects The rise time and the amplitude were measured as shown in C

	m triceps brachii				m soleus		
	A	B	C		D	E	F
Rise time (µs)	80	80	100	100	140	260	320
Amplitude (mV)	1 4	0 8	1 6	0 7	0 65	1 2	1 5

2 *Subjects and procedure*

The rise time of the spike of the motor unit potential was determined in the soleus and the lateral head of the brachial triceps muscles of 10 normal subjects 9 females and 1 male 21—46 years old (average 28 years) The subject lay supine when the brachial triceps and prone when the soleus muscle was examined With a given insertion of the electrode the subject exerted slight effort to activate one or two motor units Then the position of the electrode was adjusted by small movement (at most 1 mm) to find the steepest positive negative deflection of the spike potential without the humps or abrupt changes in slope that are seen when the spike derives from more than one fibre or a few synchronously discharging fibres (Fig 2) (Ekstedt 1964 Rosenfalck 1969) A different spike potential was obtained either by changing the depth of insertion by more than 5 mm or by inserting the electrode at a transverse distance of 5—10 mm from the preceding site In this way the rise time was recorded of 10—20 different spike potentials in each muscle The rise time was measured by extrapolating the linear portion to the positive and negative peak as illustrated in Fig 2C

The average total duration of the motor unit potentials was determined from 20—25 different motor units in each muscle The site of the electrode was changed and at each position the subject innervated with an effort which just activated 1 or 2 different motor units In the

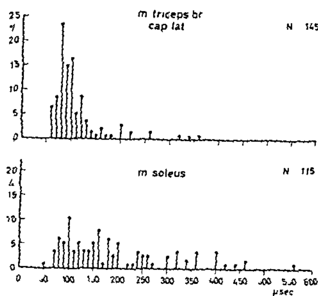


Fig 3 Distribution of rise times of motor unit potentials in the brachial triceps and in the soleus muscle of 10 normal subjects (21—46 years old)

brachial triceps muscle the average duration was based on measurements in 14 normal subject 16—37 years old in the soleus muscle on measurements in 10 other subjects 4—41 years old The average duration of motor units potentials in the brachial biceps muscle in each subject served as reference

Muscle fibre diameters were determined in the lateral head of 10 brachial triceps (6 men 4 women) and in 6 soleus muscles (5 men 1 woman) after death from traffic accidents In one woman both muscles were studied The specimens were fixed in Baker's calcium formalin 3—7 h after death embedded in gelatin and stained by Sudan Black B The major and minor diameter were measured in 200—400 muscle fibres of each muscle on cross sections 10—15 µm in thickness

Results

Typical examples of spike potentials obtained from the brachial triceps (A—C) and the soleus muscle are shown in Fig 2 (D—F) The average rise time of 115 spikes recorded in the soleus muscle was 190 ± 10 µs compared to 107 ± 4 µs for 115 different spike potentials in the lateral head of the brachial triceps ($p < 0.001$) Short rise times of 50—60 µs occurred in both muscles and the distribution was skewed toward long rise times In the soleus muscle 35% of the rise times were longer than 200 µs as compared to 5% in the lateral head of the brachial triceps (Fig 3)

Neither in the soleus nor in the brachial triceps did the rise time vary with the peak to peak amplitude of the spike potential (Fig 4) The average amplitude was the same in both muscles 1010 µV SD 811 µV ($n = 113$) in the soleus and 1007 µV SD 642 µV ($n = 137$) in the brachial triceps In the soleus muscles 9 spike potentials of high amplitude (2500—3700 µV) had rise times of 50—200 µs (Fig 5 below) An example of a spike potential of 1.5 mV and 300 µs in rise time in the

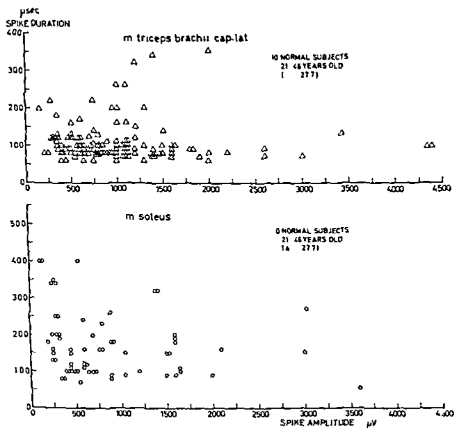


Fig 4 Rise time of spikes of motor unit potentials as a function of their peak to-peak amplitude in the lateral head of the brachial triceps and in the soleus muscles of 10 normal subjects (21—46 years old)

brachial triceps muscle is shown in Fig 5 above It is therefore unlikely that a systematically larger distance between fibre and recording electrode can account for the difference in rise times observed in the two muscles

The average total duration of motor unit potentials differs in dif rent muscles In view of the systematic differences in spike duration of motor unit potentials (rise times) found in slow and fast muscles the question arises whether those muscles whose spike durations are short also have short total duration of motor unit potentials This was not the case because the average total duration in the soleus muscle was even shorter than in the lateral head of the brachial triceps (10 4 ± 0 2 ms as compared to 12 1 ± 0 2 ms $p < 0\ 001$ $f = 22$ degrees of freedom subjects 16—40 years old)

Thus the difference in total duration between motor unit potentials in the soleus and the brachial triceps muscle recorded by conventional electromyography is determined by factors other than the rise time of the spikes of individual muscle fibres

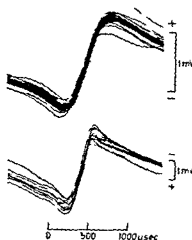


Fig. 5. Examples of motor unit spikes of high amplitude and long rise time. *Above*: in the lateral head of the brachial triceps muscle (amplitude 1.5 mV, rise time 350 µs). *Below*: in the soleus muscle (amplitude 3.9 mV, rise time 220 µs).

Muscle fibre diameters

The rate of conduction along single muscle fibres in the frog increased with increasing diameter (Håkansson 1956). It was therefore to be expected that the rise time of the spike of the action potential would shorten with increasing diameter and that the fibres in the lateral head of the brachial triceps would have a larger average diameter than those in the soleus muscle.

There was no trend for muscle fibres to be larger in diameter in the brachial triceps than in the soleus muscle. On the contrary, the diameters were larger in the soleus muscle. In the 10 brachial triceps muscles the mean diameter averaged 47 µm, S.D. 10 µm, as compared to 61 µm, S.D. 12 µm, in 6 soleus muscles. In one person both muscles were studied: the average diameter in the lateral head of the brachial triceps was 60 µm and it was 76 µm in the soleus muscle.

Discussion

The main finding of this study was that the average rise time of the spike of the motor unit potential was 80 % longer in the soleus muscle than in the lateral head of the brachial triceps and that there were some spike potentials in the soleus that had longer rise times (> 350 µs) than ever seen in the brachial triceps. Previous studies showed that the average contraction time in the soleus muscle is 66 % longer than in the lateral head of the brachial triceps and that 66 % of the fibres in the soleus have longer contraction times than ever seen in the brachial triceps (Fig. 6) (Buchthal and Schmalbruch 1970). Also in the brachial biceps muscle rise times of the spike potential and contraction times of fibre bundles were slower than in the lateral head of the brachial triceps muscle, though the difference was less marked than in the soleus muscle. In the brachial biceps Buchthal, Guld and Rosenfalck (1957) found an average rise time of 136 µs (S.D. 36 µs) for 32 spike potentials. This was 25 % longer than in the lateral head of the brachial triceps. In another study the median rise time of the spike potential of the brachial biceps muscle was

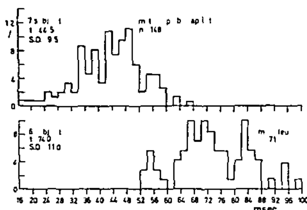


Fig 6 Distribution of contraction times of small fibre bundles in m triceps brachii (above) and m soleus of normal subjects Intramuscular temperature 36—37 C (Buchthal and Schmalbruch 1970)

112 μs (Ekstedt 1964) 20 % longer than the median rise time found in the lateral head of the brachial triceps in our experiments (92 μs) In the brachial biceps muscle 30 % of the fibre bundles have contraction times of 60 ms or longer as compared to 2 % in the lateral head of the brachial triceps (Buchthal and Schmalbruch 1969) In addition the previous studies showed a clear relation between slow contraction times and the incidence of fibres rich in mitochondria (type C fibres) on the one hand between fast contraction times and the incidence of fibres with a moderate (type B) or poor (type A) amount of mitochondria on the other In the soleus muscle of man more than 90 % of the fibres are rich in mitochondria 25 % in the brachial biceps and 2 % in the lateral head of the brachial triceps (Schmalbruch 1970)

Simultaneous intracellular and extracellular recordings from a single muscle fibre show that the rise time of the extracellular spike corresponds to the phase of active depolarisation of the surface membrane (Buchthal and Sten Knudsen 1959 Werman 1960 Murakami Watanabe and Tomita 1961) Hence the slower rise time of the spikes of the soleus muscle indicates a more protracted phase of depolarisation than in the brachial triceps muscle A slower conduction velocity along the fibres of the soleus than of the brachial triceps muscle would be associated with a longer rise time of the spike potential Whether or not conduction is slower along the fibres of the soleus than along the fibres of the brachial triceps is not known Were it demonstrated it would not be due to a smaller diameter of fibres of the soleus muscle than of the brachial triceps muscle In fact the diameter was slightly larger in the soleus than in the lateral head of the brachial triceps That the difference between the two muscles in different subjects was greater than in the person in whom both muscles were examined is probably due to the fact that the biopsies of the brachial triceps were obtained from 6 men and 4 women and in the soleus from 5 men and 1 woman In biopsies from other normal skeletal muscles the mean diameter in females was 45 μm S D 6 μm and in males 55 μm S D 5 μm (muscles from 16 females and 11 males Vilppula 1972)

The longer rise time of the spike potentials of the soleus than of the lateral head of the brachial triceps then indicates that the excitatory membrane of the slow fibres of the soleus muscle has specific properties different from those of the fast fibres in the brachial triceps Whether this difference reflects different rates of conduction or a different extent of the loops of the action currents along the fibre remains an open question

The longer rise time of the spike of the motor unit potentials in the soleus than in *the lateral head of the brachial triceps* was not reflected in a corresponding difference in mean total duration of 20—30 potentials sampled randomly from different motor units In fact in the brachial triceps the muscle with the shortest rise times the average duration was even slightly longer One might argue that the lack of a correlation between rise time and total duration of motor unit potentials was due to the fact that weak effort recruited only motor units containing slow fibres the first ones to be activated during voluntary contraction This explanation is however not correct The technique of sampling the population of motor units consists in recording while effort is exerted that just suffices to activate one or two motor units near the recording electrode The effort required to activate motor units may vary considerably for different sites of insertion from about 1 to 20 % of maximum Hence also motor units consisting of fast fibres with a high threshold are surely recruited Therefore a factor other than the rise time must account for the difference in total duration of the motor unit potentials We have previously shown that the total duration of motor unit potentials is mainly due to spatial dispersion of the motor endplates along the muscle resulting in temporal dispersion of conduction along different fibres of the motor unit (Buchthal Guld and Rosenfalck 1955)

We wish to thank Drs L Bonomi P Hubbe and D Kokkini for permission to use their data on duration of motor unit potentials Dr H Schmalbruch for permission to use his cross sections of normal muscles and Mrs Birte Duborg for technical assistance
The work was supported by grants from the Muscular Dystrophy Associations of America New York and the Michaelsen Foundation Copenhagen

References

BABKIN B P Zeigen die Aktionsströme verschieden rasch zuckender Muskeln des Frosches einen verschiedenen zeitlichen Verlauf? *Pflüger s Arch ges Physiol* 1908 125 595 600
BUCHTHAL F C GULD and P ROSENFALCK Innervation zone and propagation velocity in human muscle *Acta physiol scand* 1955 35 174—190
BUCHTHAL F C GULD and P ROSENFALCK Volume conduction of the spike of the motor unit potential investigated with a new type of multielectrode *Acta physiol scand* 1958 38 331—354
BUCHTHAL F and O STEN KNUDSEN Impulse propagation in striated muscle fibers and the role of the internal currents in activation *Ann N Y Acad Sci* 1959 81 422—445
BUCHTHAL F and H SCHMALBRUCH Spectrum of contraction time of different fibre bundles in the brachial biceps and triceps muscles of man *Nature (Lond)* 1969 222 89
BUCHTHAL F and H SCHMALBRUCH Contraction time and fibre types in intact human muscle *Acta physiol scand* 1970 79 435—452
BULLER A J D M LEWIS and R M A P RIDGE Some electrical characteristics of fast twitch and slow twitch skeletal muscle fibres in the cat *J Physiol (Lond)* 1965 180 29 30 P
ECCLES J C and W J O'CONNOR Responses which nerve impulses evoke in mammalian striated muscles *J Physiol (Lond)* 1939 97 44 102

EKSTEDT J Human single muscle fiber action potentials *Acta physiol scand* 1964 *61* Suppl 226 pp 1—96

HÅKANSSON C H Conduction velocity and amplitude of the action potential as related to circumference in the isolated muscle fibre of frog muscle *Acta physiol scand* 1956 *37* 14—34

HENNEMAN E and C B OLSON Relation between structure and function in the design of skeletal muscles *J Neurophysiol* 1965 *28* 581—598

KOHLRAUSCH A Über das Elektromyogramm roter und weisser Muskeln *Arch Anat Physiol* Physiol Abt 1912 283—295

LEWIS D M The effect of denervation on the mechanical and electrical responses of fast and slow mammalian twitch muscle *J Physiol* (Lond) 1972 *222* 51—75

LORKOVIĆ H Membrane potentials and mechanical tension in white and red muscle *Amer J Physiol* 1971 *221* 1044—1050

MCPHEDRAN A M R B WUERKER and E HENNEMAN Properties of motor units in a homogeneous red muscle (soleus) of the cat *J Neurophysiol 1965 28 71—84*

MURAKAMI M K WATANABE and T TOMITA Effect of impalement with a microelectrode on the local cell membrane Study by simultaneous intra and extracellular recording from the muscle fibre and giant axon *Jap J Physiol* 1961 *11* 80—88

NISSEN PETERSEN H C GULD and F BUCHTHAL A delay line to record random action potentials *Electroenceph clin Neurophysiol 1969 26 100—106*

ROSENFALCK P Intra and extracellular potential fields of active nerve and muscle fibres *Acta physiol scand* 1969 Suppl *321* pp 1—168

SCHMALBRUCH H Die quergestreiften Muskelfasern des Menschen *Ergebn Anat Entwickl Gesch* 1970 *43* 1—73

STUART D G G E GOSLOW JR and R L GERLACH Motor unit properties of cat hind limb *muscles Abstr 4 Int Congr of EMG Brussels Sept. 1971 p 139—140*

WERMAN R Electrical inexcitability of the synaptic membrane in the frog skeletal muscle fibre *Nature* (Lond) 1960 *188* 149—150

VILPPULA A Muscular disorders in some collagen diseases *Acta med scand* 1972 Suppl 540 pp 1—47

WUERKER R B A M MCPHEDRAN and E HENNEMAN Properties of motor units in a heterogenous pale muscle (m gastrocnemius) of the cat *J Neurophysiol* 1965 *28* 85—99

Acta physiol scand. 1973 87 270—276

From Burn Center Dept of Plastic Surgery University Hospital Uppsala, Dept. of Chemistry Karolinska Institutet Stockholm Sweden

Prostaglandins in Human Burn Blister Fluid

By

Gosta Arturson Mats Hamberg and Carl Evert Jonsson

Received 30 May 1972

Abstract

Arturson, G M Hamberg and C E Jonsson *Prostaglandins in human burn blister fluid* Acta physiol scand 1973 87 270—276

Acidic lipids with smooth muscle stimulating activity were isolated from human burn blister fluid Analysis by silicic acid chromatography and thin layer chromatography indicated the presence of prostaglandin E and F compounds Prostaglandin E_2 (about 1 9 ng/ml of blister fluid) was identified and quantitatively determined by gas liquid chromatography—mass spectrometry No metabolic conversion of tritium labelled prostaglandin E_1 incubated with blister fluid was observed

Many pharmacologically active compounds have been implicated in burn injury (for review see Spector and Willoughby 1968) Local vascular reactions have been supposed to be mediated by vasoactive compounds released or formed in the injured tissue An efflux of these into the systemic circulation would partly explain general symptoms of the burn syndrome Recently it was shown (Jonsson 1971 Änggård and Jonsson 1971 Hamberg and Jonsson 1972) that scalding in dogs and guinea pigs results in increased biosynthesis of prostaglandins and a release of these into lymph draining the scalded tissues

Prostaglandins have many pharmacological activities (for review see Bergström Carlson and Weeks 1968 Hinman 1972 Horton 1972 Weeks 1972) some of which are of interest in connexion with burn injury Since prostaglandin E-compounds cause erythema due to vasodilatation when injected in man (Solomon Juhlin and Kirschenbaum 1968 Juhlin and Michaelsson 1969 Crunkhorn and Willis 1971) and promotes leucotaxis (Kaley and Weiner 1971) they have the pharmacological properties of a mediator of inflammation

Previously it was shown that human skin has the capacity both for formation and metabolism of prostaglandins (Jonsson and Änggård 1972) The present study reports the presence of prostaglandins in human burn blister fluid indicating an increased biosynthesis of prostaglandins in burn injury in man Part of this work has earlier been published in preliminary form (Änggård Arturson and Jonsson 1970)

Material

Burn blister fluid was obtained from patients with skin burns admitted to the hospital The patients required treatment with i v fluids analgetics and sedatives 2 h to several days following the burn injury the blisters were punctured with a sterile needle and the contents aspirated in polyethylene syringes and transferred to plastic bottles containing 2—5 vol of absolute ethanol with d l α tocopherol (0 1 %) as antioxidant and stored at −20 C before processing For studies on the metabolism of ^3H labelled prostaglandin E_1 (PGE_1) the fluid was collected (without ethanol) 2—4 h after burn injury and kept at −20 C before use Fluid was discarded if the gross appearance of the blister suggested infection

Unlabelled prostaglandins and [3 3 4 4 D_4] prostaglandin E_2 (PGE_2) was kindly supplied by the Upjohn Company Kalamazoo Mich Tritium labelled prostaglandin E_1 (PGE_1) was purchased from New England Nuclear Corp Boston Mass The specific activity was 25 or 87 3 Ci/mmole Tritium labelled PGE_2 (specific activity 4 5 Ci/mmole Änggård Grèen and Samuelsson 1965) and prostaglandin F (PGE$_{2\alpha}$ specific activity 55 / Ci/mmole Granström Inger and Samuelsson 1965) was obtained as earlier described

Methods

Acidic lipid extracts of burn blister fluid were prepared for recording smooth muscle stimulating activity by a method previously described (Änggård and Jonsson 1971) The blister ethanol mixture was filtered and the clear filtrate reduced to a small volume under reduced pressure at room temperature After extraction with heptane the pH of the aqueous solution was adjusted to 3 with 1 N HCl and extracted 3 times with equal volumes of ether The combined ether phases were shaken with 0 1 M phosphate buffer pH 8 The water phase was acidified to pH 3 by the addition of 3 N HCl and extracted twice with ether The combined ether phases were washed with small volumes of water until neutral reaction and evaporated to dryness The recovery of ^3H PGE_2 (0 1 μCi 8 ng) added to the blister ethanol mixture was about 75

For characterization and identification of prostaglandins in pooled blister fluid a slightly modified extraction procedure was used The mixture was filtered the filtrate washed with ethanol and the combined ethanol extracts reduced to a small volume After extraction with petroleum ether (bp below 40 C) the water phase was acidified to pH 3 with 2 N HCl extracted twice with ether and the combined ether extracts evaporated *in vacuo* after washing with small amounts of water until neutral reaction.

Blister fluid incubation In two experiments 2 and 8 ml blister fluid was incubated for 30 and 120 min respectively at 37 C with ^3H PGE_1 (0 5 μCi 7 ng) NAD (10^{-3} M) and dithiotreitol (10^{-3} M) The incubations were terminated by the addition of ethanol and extracts of acidic lipids were obtained as described above This material was subjected to reversed phase partition chromatography after addition of 0 5 mg of unlabelled PGE_1

Chromatographic methods Silicic acid chromatography of lipid extracts containing ^3H labelled PGE_1 was carried out essentially as earlier described (Bygdeman and Samuelsson 1966) Columns of 1 g silicic acid (Unisil 100—200 mesh Clarkson Chemical Corp Williamsport Penns) were used Elution was accomplished by a linear gradient starting with ethyl acetate benzene (1 9 v/v) and ending with ethyl acetate After this the columns were eluted with 15 ml of methanol Fractions of 1 5 ml were collected and assayed for radioactivity Aliquots of pooled fractions were taken to dryness and assayed for smooth muscle stimulating activity with the colon of the gerbil

Thin layer chromatography was performed essentially as described by Green and Samuelsson (1964) The lipid extracts containing ^3H PGE_1 and ^3H PGF were applied on a 12 cm line and the plates developed in solvent system A I Zones of about 1 cm were scraped off and eluted with 3×1 ml of ethanol The filtered eluates were taken to dryness and assayed for radioactivity and smooth muscle stimulating activity with colon of the gerbil

Reversed phase partition chromatography was performed as earlier described (Norman and Sjövall 1958) Solvent system C 1 was used Aliquots of the fractions were assayed for radioactivity PGE_1 was detected after treatment of aliquots of the fractions with 0 5 N ethanolic NaOH followed by spectrophotometric determination of the chromophore formed (PGB_1) at 280 nm (Bergström *et al* 1963)

Bioassay Smooth muscle stimulating activity was recorded on colon of the gerbil and rat fundus strip (Weeks Schultz and Brown 1968) The tissues were mounted in a 1 ml temperature controlled bath bubbled with oxygen PGE_2 was used as reference When testing biological extracts organic solvents were evaporated and the organic material reconstituted in Tyrode s or de Jalon s solution with 10 % ethanol

Measurement of radioactivity Radioactivity was determined in a Packard liquid scintillation spectrometer (model 3320) As scintillator 10 ml of Instagel (Packard Instr) was used Corrections for counting efficiency were made by the use of external standardization

TABLE I

Patient no	Hours after burn injury						
	2	6	8	12	24	36	60
1	0 5						
2		2 6		1 2			
3			1 3				
4					3 6	0 3	0 2

Smooth muscle stimulating activity in acidic lipid extracts of burn blister fluid from 4 patients Biological activity was recorded on rat fundus strip and is expressed in terms of ng of PGE per ml blister fluid after correction for losses due to extraction

Results

Acidic lipid extracts obtained from blister fluid collected 2 to 60 h after injury in 4 patients were assayed for smooth muscle stimulating activity on rat fundus strip The results are shown in Table I The mean biological activity was equivalent to 1 4 ng PGE_2 per ml blister fluid (range 0 2—3 6) The highest values were observed during the first 24 h after the burn injury

For further identification of the smooth muscle stimulating material lipid extracts from pooled blister fluids were subjected to silicic acid chromatography, a procedure known to afford separation of prostaglandin E and F compounds (Samuelsson 1963) On silicic acid column chromatography one peak of smooth muscle stimulating material appeared This material coincided with the added ^{3}H PGE_1 (Fig 1) However the peak of smooth muscle stimulating material showed considerable tailing probably due to the presence of prostaglandin F compounds

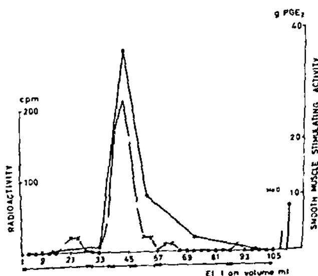


Fig 1 Silicic acid chromatography of 35 % of a lipid extract obtained from 74 ml of blister fluid The extract applied to the column contained biological activity equivalent to about 56 ng PGE2 Fractions were pooled as indicated by arrows at bottom and assayed for smooth muscle stimulating activity on colon of the gerbil (——●——) Radioactivity due to added ^{3}H PGE1 (0 1 μCi 0 4 ng) was recorded in aliquots of the fractions (— — x — —)

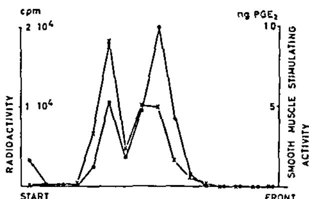


Fig 2 Thin layer chromatography of 95 c of a lipid extract obtained from 67 ml of blister fluid The extract contained biological activit equivalent to about 28 ng PGE Smooth muscle stimulating activity on colon of the gerbil (——●——) and radioactivity (——×——) of 1 cm zones were determined Smooth muscle stimulating activity is expressed in terms of PGE_2 and the contribution due to added tracers (^{3}H PGE_1 0 3 μCi and ^{3}H PGF 0 3 μCi smooth muscle stimulating activities in terms of PGE_2 about 1 ng and 9 ng respectively) have been substracted

On thin layer chromatography (Fig 2) 2 peaks of smooth muscle stimulating activity were found These coincided with the added ^{3}H PGE_1 (R_f about 0 5) and ^{3}H PGF_1 (R_f about 0 3)

Identification and quantitative determination of PGE in human burn blister fluid was performed with an isotope dilution technique recently described (Axen *et al* 1971) [3 3 4 4 $^2H_4$,17 18 ^{3}H] PGE 5 7 μg was added to 70 ml of blister fluid The lipid extract was subjected to reversed phase partition chromatography with solvent system C 1 (Norman and Sjovall 1958) Material in the peak of tritium labelled PGE was isolated by extraction with diethyl ether and subsequently treated in succession with diazomethane O methyl hydroxylamine hydrochloride in pyridine and acetic anhydride in pyridine (*cf* Green 1969) After purification by silicic acid chromatography (elution with ethyl acetate benzene (2 8 v/v)) the derivative was subjected to gas liquid chromatographic mass spectrometric analysis The mass spectrum recorded on the major peak of the derivative (*cf* Green 1969) showed ions of high intensity at m/e 423 (M−60 loss of CH_3COOH), 392 (M−(60+31) loss of CH_3COOH plus OCH_3) and 332 (M−(2×60+31) loss of 2 CH_3COOH plus OCH_3) as expected for the methyl ester O methyloxime acetate derivative of [3 3 4 4 H_4] PGE (Axen *et al* 1971)

However ions at m/e 419 and 388 were also seen demonstrating the presence of unlabelled PGE The ratio of unlabelled to tetradeuterated molecules was 0 033 as determined by multiple ion analysis (m/e 419 and m/e 423 *cf* Axen *et al* 1971) Since the standard of deuterium labelled PGE_2 contained 1 0 % unlabelled molecules the found value showed that 5700 × 0 023 ng = 131 ng of PGE was present in the sample of blister fluid The calculated concentration was thus 1 9 ng/ml

In 2 expts tritium labelled PGE_1 was incubated with 2 and 8 ml respectively of burn blister fluid On reversed phase partition chromatography of acidic lipid extract only one peak of radioactivity appeared This peak coincided with that of added ... PGE₁ showing that no transformation of the incubated tritium labelled ... of added ...

Discussion

Material with smooth muscle stimulating activity has earlier been demonstrated in human burn blister fluid but the identity of the active compound(s) has not been established Lewis and Grant (1924) and Harris (1929) described histamine like activity in human burn blister and estimated the amount in burn blister fluid to be equal to that in blood Armstrong *et al* (1957) reported the presence of a pain producing substance with pharmacological activities formed in blister fluid after glass contact This activity was supposed to be due to *in vitro* formation of a polypeptide Goodwin *et al* 1963 also described biological activity in human burn blister and ascribed this activity to polypetides of the kinin system Johansson (1960) claimed the presence of serotonin (5 hydroxy tryptamine) in concentration of 0 1 μg/ml

Our results demonstrate the presence of smooth muscle stimulating acidic lipids in human burn blister fluid The chromatographic data indicated that this activity was due to prostaglandins of the E and F series Assays on rat fundus strips showed that the smooth muscle stimulating activity was equivalent to 1—2 ng of PGE_2 per ml blister fluid PGE_2 was unequivocally identified by a mass spectrometric method (Axen *et al* 1971) The amount of PGE_2 as determined by this method was 1 9 ng/ml of blister fluid

The estimated concentration of prostaglandins in burn blister fluid cannot be compared to plasma levels as reliable data for prostaglandins in plasma are not yet available (*cf* Green *et al* 1972) However it is of interest that prostaglandin E compounds exert biological effects in extremely low doses *e g* the threshold dose for vasodilatation is about 0 1—0 3 ng/ml (Fredholm Öberg and Rosell 1970, McGiff *et al* 1969 1970) For effects on erythrocyte filtrability even lower doses are required (Allen and Rasmussen 1971)

It has recently been shown that after scalding of the dog paw prostaglandins are released into lymph draining the tissue (Anggard Arturson and Jonsson 1970 Jonsson 1971 Anggard and Jonsson 1971) After scalding injury to the guinea pig there is an increased biosynthesis of prostaglandins with high content of prostaglandins at the site of injuried skin and increased urinary excretion of the main metabolite of PGE_1 and PGE (Hamberg and Jonsson 1972) Therefore the occurrence of prostaglandins in human burn blister fluid probably reflects an increased biosynthesis in skin following burn injury It has earlier been shown that homogenates of human skin are capable of prostaglandin biosynthesis (Jonsson and Ånggård 1972)

Prostaglandins have also been demonstrated in inflammatory exudates. In carrageenan induced inflammatory exudates in rats PGE_2 accounts for the major part of the smooth muscle stimulating activity (Willis 1969 1970) In man Greaves Söndergard and McDonald Gibson (1971) demonstrated a mixture of prostaglandin E_2 and F-compounds following perfusion of inflammed skin of patients with allergic contact eczema

Burn blister fluid can be considered as an ultrafiltrate of plasma formed by increased microvascular permeability for macromolecules (Arturson 1961) as well as increased transcapillary filtration pressure and increased tissue osmotic forces (Arturson and Mellander 1964) after burn injury PGE compounds have vasodilatory effects and increase capillary permeability in man (Solomon Juhlin and Kirschenbaum 1968 Juhlin and Michaelsson 1969 Crunckhorn and Willis 1971) and promote leucocyte migration (Kaley and Weiner 1971) Thus an increased formation of prostaglandins in human skin as evidenced by the release of prostaglandins into burn blister fluid may partly explain vasodilatation increased microvascular permeability and accumulation of polymorphonuclear leucocytes following burn injury (Sevitt 1957)

This work was supported by grants by the Swedish Medical Research Council (projects no 13X 2828 and 40X 676) The skilful techniqual assistance of Mrs M Eriksson and Mrs V Lagerstrom and Miss K Wahlberg is greatfully acknowledged

References

Allen J E and H Rasmussen Prostaglandin and isoproterenol induced changes in the red blood cell membrane *Clin Res* 1971 *19* 559

Anggard E G Arturson and C E Jonsson Efflux of prostaglandins in lymph from scalded tissues *Acta physiol scand* 1970 *80* 46A—47A

Anggard E K Green and B Samuelsson Synthesis of tritium labelled prostaglandin E_2 and studies on its metabolism in guinea pig lung *J biol Chem* 1965 *240* 1932—1940

Anggard E and C E Jonsson Efflux of prostaglandins in lymph from scalded tissue *Acta physiol scand* 1971 *81* 440—447

Armstrong D J B Jepson C A Keele and J W Stewart Pain producing substance in human inflammatory exudates and plasma *J Physiol* (Lond) 1957 *135* 350—370

Arturson G Pathophysiological aspects of the burn syndrome *Acta chir scand* 1961 Suppl 274

Arturson G and S Mellander Acute changes in capillary filtration and diffusion in experimental burn injury *Acta physiol scand* 1964 *62* 457—463

Axen U K Green D Horlin and B Samuelsson Mass spectrometric determination of picomole amounts of prostaglandins E_2 and $F_{2\alpha}$ using synthetic deuterium labelled carriers *Biochem biophys Res Commun* 1971 *45* 519—525

Bergstrom S L A Carlson and J R Weeks The prostaglandins a family of biologically active lipids *Pharmacol Rev* 1968 *20* 1—48

Bergstrom S R Ryhage B Samuelsson and J Sjovall The structures of prostaglandin E F and F β *J biol Chem* 1963 *238* 3555—3564

Bygdeman M and B Samuelsson Analyses of prostaglandins in human semen *Clin chim Acta* 1966 *13* 465—474

Crunkhorn P and A L Willis Cutaneous reactions to intradermal prostaglandins *Brit J Pharmacol* 1971 *41* 49—56

Fredholm B B B Öberg and S Rosell Effects of vasoactive drugs on circulation in canine subcutaneous adipose tissue *Acta physiol scand* 1970 *79* 564—574

Goodwin L G C R Jones W H G Richards and J Kohn Pharmacologically active substances in the urine of burned patients *Brit J exp Path* 1963 *44* 551—560

Granstrom E U Inger and B Samuelsson The structure of a urinary metabolites of prostaglandin F_1 in the rat *J biol Chem* 1965 *240* 457—461

Greaves M W J Sondergaard and W McDonald-Gibson Recovery of prostaglandins in human cutaneous inflammation *Brit med J* 1971 2 258—261

Green K Gas chromatography mass spectrometry of O methyloxime derivates of prostaglandins *Chem phys Lip* 1969 *3* 254—272

Green K F Beguin M Bygdeman M Toppozada and N Wiqvist Analysis of prostaglandin F_2 and metabolites following intravenous intraamniotic and vaginal administration of F *WHO report* *r 2 n prostaglandins* Stockholm 1972 189—200

GREEN K. and B SAMUELSSON Thin layer chromatography of prostaglandins *J Lipid Res* 1964 *5* 117—120
HAMBERG M and C E JONSSON Increased synthesis of prostaglandins in the guinea pig following scalding injury *Acta physiol scand* 1973 *87* 240—245
HARRIS K E Observations upon a histamine like substance in skin extracts *Heart* 1929 *14* 337—367
HINMAN J W Prostaglandins *Ann Rev Biochem* 1972 In press
HORTON E W *Prostaglandins* Springer Verlag Berlin Heidelberg New York 1972
JOHANSSON S A 5 hydroxytryptamine in burns *Acta physiol scand* 1960 *48* 126—131
JONSSON C E Smooth muscle stimulating lipids in peripheral lymph after experimental burn injury *Scand J plast Reconstr Surg* 1971 *5* 1—5
JONSSON C E and E ANGGARD Biosynthesis and metabolism of prostaglandin E_2 in human skin *Scand J clin Lab Invest* 1972 *29* 289—296
JUHLIN L and G MICHAELSSON Cutaneous vascular reactions to prostaglandins in healthy subjects and in patients with urticaria and atopic dermatitis *Acta derm venerol* (Stockh) 1969 *49* 251—261
KALEY G and R WEINER Effect of prostaglandin E_1 on leucocyte migration *Nature New Biol* 1971 *234* 114—115
LEWIS T and R T GRANT Vascular reactions of the skin to injury II The liberation of histamine like sub tance in injured skin, the underlying cause of factitious urticaria and of wheals produced by burning and the observations upon the nervous control of certain skin reactions *Heart* 1924 *11* 209—265
MCGIFF J C K CROWSHAW N A TERRAGNO and A J LONIGRO Renal prostaglandins possible regulators of the renal actions of pressor hormones *Nature* (Lond) 1970 227 1255—1257
MCGIFF J C N A TERRAGNO J C STRAND J B LEE A J LONIGRO and K K F NG Selective passage of prostaglandins across the lung *Nature* (Lond) 1969 223 744—745
NORMAN A and J SJOVALL On the transformation and enterohepatic circulation of cholic acid in the rat *J biol Chem* 1958 *233* 872—885
SAMUELSSON B Isolation and identification of prostaglandins from human seminal plasma *J biol Chem* 1963 *238* 3229—3234
SEVITT S *Burns Pathology and therepeutic applications* London Butterworth & Co Ltd 1957
SOLOMON L M L JUHLIN and M B KIRSCHENBAUM Prostaglandins on cutaneous vasculature *J invest Derm* 1968 *51* 280—282
SPECTOR, W G and D A WILLOUGHBY *The pharmacology of inflammation* The English University Press Ltd London 1968
WEEKS J R Prostaglandins *Ann Rev Biochem* 1972 In press
WEEKS J R J R SCHULTZ and W E BROWN Evaluation of smooth muscle bioassays for prostaglandins E_1 and $F_{1\alpha}$ *J appl Physiol* 1968 25 783—785
WILLIS A L Parallel assay of prostaglandin like activity in rat inflammatory exudate by means of cascade superfusion *J Pharm Pharmacol* 1969 *21* 126—128
WILLIS A L Identification of prostaglandin E_2 in rat inflammatory exudate *Pharmac Res Commun* 1970 *2* 297—304

Acta physiol scand 1973 87 277—282
From the Department of Physiology University of Goteborg Sweden

Intestinal Vasodilatation in Response to Transmural Electrical Field Stimulation

By

BJORN BIBER JOHN FARA and OVE LUNDGREN

Received 5 June 1972

Abstract

BIBER B J FARA and O LUNDGREN *Intestinal vasodilatation in response to transmural electrical field stimulation* Acta physiol scand 1973 87 277—282

Electrical field stimulation of the small intestine *in vivo* in cats induces a 60—150 % increase in total intestinal blood flow The vasodilatory response to field stimulation is blocked by tetrodotoxin and lidocaine but is unaffected by extrinsic denervation of the intestine by cholinergic and adrenergic pharmacological blocking agents and also by cross perfusion of the stimulated intestine from a donor animal The participation of a local nervous structure in the control of intestinal blood flow is suggested

The mechanisms involved in the local control of intestinal blood flow are as yet not fully known However in recent experiments on cats it was shown that mechanical stimulation of the mucosa in a denervated jejunal segment caused a doubling of intestinal blood flow (Biber Lundgren and Svanvik 1971) Furthermore nerve conductivity blocking agents such as tetrodotoxin and lidocaine given locally to the intestine completely abolished the vasodilator response to mechanical stimulation (Biber *et al* 1971) These findings suggest the existence of an intramural nervous mechanism engaged in intestinal blood flow control

While it is well established that transmural electrical field stimulation across the intestinal wall activates nervous structures involved in the control of intestinal motility (Paton 1955 Bennet *et al* 1966 Bulbring and Tomita 1967) there are apparently no reports concerning field stimulation and intestinal blood flow Since the earlier study on mechanical stimulation suggested the existence of a local nervous mechanism affecting blood flow the present study was undertaken to investigate possible vascular effects induced by transmural electrical field stimulation of the cat jejunum



Fig 1



Fig 2

Fig 1 Inner electrode Plastic tube with wound silver wire Outer diameter 5 mm
Fig 2 Outer electrode of flexible silver wire Diameter 20 mm

Methods

A Operative procedures The experiments were performed on 16 cats of both sexes anesthetized with chloralose i v (50—70 mg/kg b w) after ether induction The animals were deprived of food for at least 24 h prior to the experiment

The operative procedures were similar to those previously described in detail (*e g* Biber *et al* 1971) A jejunal segment and its lymph nodes together weighing 15—25 g was chosen for the experiment and the rest of the intestine extirpated Venous outflow was recorded by means of an optical drop recorder operating an ordinate writer Arterial blood pressure was monitored from the left femoral artery *via* a Statham pressure transducer (P23 AC) Blood pressure and flow were continuously recorded on a Grass polygraph In some experiments the jejunal segment was cross-perfused from another cat *via* a siliconized cannula, connecting the femoral artery of the donor to the superior mesenteric artery of the recipient The venous out flow from the cross-perfused intestine was returned to the donor *via* its jugular vein

The nerves along the superior mesenteric artery were cut and in most experiments the splanchnic nerves as well In some cases the left adrenal gland was denervated and the right one excluded from the circulation by ligatures

B Experimental procedures To perform an electrical field stimulation across the intestinal wall a soft plastic tube (outer diameter 2 or 5 mm) with a flattened silver wire wound around its full length (Fig 1) was cautiously inserted into the jejunal lumen serving as the inner electrode and held in position by ligatures at the 2 ends of the segment The tube was pierced by numerous holes so that measurements of intestinal luminal pressure could be performed if desired

The jejunal segment with its luminal tube was placed into a triangular lucite chamber and a specially designed silver wire electrode (Fig 2) was positioned around the outside of the intestine The chamber was filled with Tyrode solution covered with a plastic sheet and placed in the abdomen of the cat thus keeping the intestine *in situ* The temperature inside the chamber was maintained at 38 C controlled by an infrared lamp and a thermocouple in the bath

The outer and inner electrodes were connected to a Grass S 5 stimulator delivering pulses of constant voltage Stimulations of long duration and high frequency were limited by the fact that the Grass S 5 stimulator could not produce pulses with a duration exceeding that of the interval between the stimuli For such procedures a stimulator constructed at the department was therefore used the stimuli intermittently being checked by means of an oscilloscope Pulses at constant current strength were delivered from a specially designed constant current generator operated through the stimulators. The electrical resistance over the electrodes was continuously measured during the stimulation periods making it possible to detect any accidental short circuiting between them. The stimulation characteristics used are described in Results.

C Drugs Atropine sulphate was given i v and other drugs, administered into the superior mesenteric artery *via* a cannula retrogradely inserted into one of its branches were propranolol (Inderal® ICI) phenoxybenzamine (Dibenyline® Smith Kline & French Laboratories Ltd) isopropylnoradrenaline sulphate tetrodotoxin (Calbiochem Luzern Switzerland) and lidocaine (Xylocaine® Astra 2 % solution)

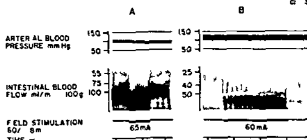


Fig 3 The effect of electrical transmural field stimulation with constant current on total intestinal venous outflow Panels A and B show typical experiments in two cats

Results

A Constant current Using the inner electrode as cathode electrical field stimulation with pulses of constant current caused an intestinal blood flow increase of 60—150 % above control evident within 5 s and usually fully developed within 10—15 s (Fig 3) Blood flow remained increased at an almost constant level during the entire stimulation period but returned to control within 20 s after cessation The field stimulations were usually delivered for about 1 min however the vasodilatory response could be maintained during stimulation periods of 10 min or more Detailed studies on 5 cats showed that pulses with a current strength of 60—80 mA a frequency of around 60 Hz and a pulse duration of 8—10 ms were the optimal electrical stimuli for inducing a maximal blood flow increase (Fig 4) Spontaneous intestinal motility, measured as intraluminal pressure changes was transiently inhibited during the field stimulation periods No other changes in intraluminal pressure were observed during stimulation

B Constant voltage Field stimulation with pulses of constant voltage caused increases of intestinal blood flow that were initially very similar to those described above The maximal response was obtained by 4—6 V 8—10 ms and 60—80 Hz However this blood flow increase usually lasted for only 2—3 min after which flow gradually returned towards control despite continued stimulation Electrical resistance between the electrodes measured during the stimulation periods showed a continuous increase eventually reaching such high levels that little if any current passed The increase of resistance was probably due to polarization of silver electrodes in the Tyrode solution The motility response evoked by pulses of constant voltage was similar to that described above

C Blocking drugs Atropine (1 mg/kg b w) given during 10 expts did not affect the intestinal blood flow responses to transmural stimulation

In 5 expts the α receptor blocker phenoxybenzamine (2 mg/kg b w) was given Its effectiveness was tested by the disappearance of the resistance increase normally induced by direct vasoconstrictor fibre stimulation In 4 expts the β-receptor blocker propranolol (1 mg/kg b w) was given and its effectiveness was shown by the ...

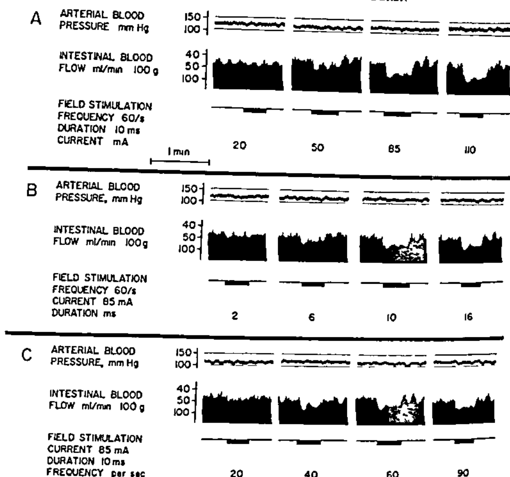


Fig 4 Intestinal blood flow in response to field stimulation with pulses of increasing current (Panel A) duration (Panel B) and frequency (Panel C) showing threshold for inducing vasodilatation Maximal blood flow increase is reached at a stimulation current of 85 mA a duration of 10 ms and a frequency of 60 Hz

abolishment of the intestinal vasodilator response to isopropylnoradrenaline In all these experiments the vasodilator response to field stimulation was completely unaltered as compared to control

In 6 experiments tetrodotoxin (2—6 μg/kg b w) was given to block nervous conductivity within the intestinal preparation Nerve blockade was tested by electrical vasoconstrictor fiber stimulation Immediately following each tetrodoxin injection a virtually complete abolishment of the vasodilator response to field stimulation was seen In 5 of the experiments it was possible to maintain the animal for up to several hours after tetrodotoxin administration during which time the vascular response to field stimulation and vasoconstrictor fibre stimulation gradually returned to control

Lidocaine a local anesthetic affecting nerve conductivity was repeatedly given i.a to the intestinal preparation in 6 expts Nerve blockade was tested as with

tetrodotoxin A complete or nearly complete abolishment of the vasodilator response to transmural field stimulation was observed immediately after lidocaine administration Within 15—30 min the response to vasoconstrictor nerve fiber stimulation had returned whereas the response to field stimulation remained blocked for 30 min to 1 h

Discussion

Electrical field stimulation applied across the intestinal wall induces an excitation of local nervous structures It has for example been shown that such stimulation can excite both inhibitory (Holman and Hughes 1965) and excitatory (Campbell 1966) nerve fibers influencing the intestinal smooth muscle layers However some observations indicate that transmural field stimulation may induce a direct hyperpolarization and relaxation of intestinal smooth muscle independent of the innervation (Bulbring *et al* 1965)

In the present study vascular smooth muscle relaxation has been demonstrated in response to transmural field stimulation of the intestine This vasodilator response was constantly blocked by tetrodotoxin and lidocaine Tetrodotoxin has previously been shown to block conductivity without *per se* interfering with smooth muscle function (Gershon 1967) Additionally in the present experiments lidocaine blocked the response to electrical field stimulation without altering vascular reactivity as tested by vasoconstrictor fiber stimulation within the 15 min period after total blockade Therefore it is unlikely that the intestinal vasodilator response to field stimulation was caused by direct hyperpolarization of the vascular smooth muscles but rather is suggested to be mediated through some neurogenic vasodilator influence

As the intestinal segments were extrinsically denervated and in the cross perfusions separated from the recipient extrinsic nervous connections are obviously not essential for this neurogenic vasodilator structure Further the experiments using α and β adrenergic and cholinergic receptor blockers strongly suggest that the vasodilator effect is mediated by receptors of a different nature

Previous investigations (Biber *et al* 1971) have demonstrated that mechanical stimulation of the intestinal mucosa can induce a vasodilatation that is evidently mediated through local nervous structures unaffected by adrenergic or cholinergic blocking drugs Thus findings so far point to the existence of a common local nervous mechanism controlling intestinal blood flow The precise nature of this mechanism and the mediator producing the vasodilation are at present being investigated

We gratefully acknowledge the kind and skillful technical assistance of Mrs E a Bengtsson Mrs Dagr v Johansson and Miss Lisbeth Larsson We are indebted to A Johnsson & Co for generous supply of phenoxybenzamine This research was sponsored in part by the Swedish Medical Research Council (No B 72 14X 2855-03 and partly No B 72 14X 16-08A) and by the Faculty of Medicine University of Goteborg J Fara was supported by a National Institutes of Health Fellowship 1 FO? HE50 664 01 from the Heart and Lung Institute

References

BENNET M R, G BURNSTOCK and M E HOLMAN Transmission from intramural inhibitory nerves to the smooth muscle of the guinea pig taenia coli *J Physiol* (Lond) 1966 *182* 541—558

BIBER B, O LUNDGREN and J SVANVIK Studies on the intestinal vasodilatation observed after mechanical stimulation of the mucosa of the gut *Acta physiol scand* 1971 *82* 177—190

BULBRING E, H KURIYAMA and T TOMITA Discrimination between the inhibitory potential and electrotonic hyperpolarization in the smooth muscle *J Physiol* (Lond) 1965 *181* 8—10 P

BULBRING E and T TOMITA Properties of the inhibitory potential of smooth muscle as observed in the response to field stimulation of the guinea pig taenia coli *J Physiol* (Lond) 1967 *189* 299—315

CAMPBELL G Nerve mediated excitation of the taenia of the guinea pig caecum *J Physiol* (Lond) 1966 *185* 148—159

GERSHON M D Effects of tetrodotoxin on innervated smooth muscle preparations *Brit J Pharmacol* 1967 *29* 259—279

HOLMAN M E and J R HUGHES Inhibition of intestinal smooth muscle *Aust J exp Biol med Sci* 1965 *43* 277—290

PATON W D M The response of the guinea pig ileum to electrical stimulation by coaxial electrodes *J Physiol* (Lond) 1955 *127* 40—41 P

Acta physiol scand 1973 87 283—288

From the Department of Anatomy University of Helsinki Finland

Histochemically Demonstrable Catecholamines and Cholinesterases of the Rat Uterus during Estrus Cycle, Pregnancy and after Estrogen Treatment

By

ANTTI HERVONEN LASSE KANERVA and RALF LIETZEN

Received 6 June 1972

Abstract

HERVONEN A L KANERVA and R LIETZEN *Histochemically demonstrable catecholamines and cholinesterases in the rat uterus during estrus cycle pregnancy and after estrogen treatment* Acta physiol scand 1973 *87* 283—288

The changes of the adrenergic and cholinergic nerves of the rat uterus were examined during the estrous cycle, pregnancy and after estrogen treatment. During the first 2 weeks of pregnancy and after estrogen treatment the fluorescence intensity and the thickness of the extra vascular adrenergic nerve fibres increased but decreased in the end of pregnancy The activity and also the density of AChE positive nerves increased after estrogen treatment and during the first 2 weeks of pregnancy and decreased at the end of pregnancy The AChE-activity and the intensity of fluorescence in the nerves was somewhat stronger in estrus than diestrus

The myometrial cells of the rat uterus are innervated by short adrenergic neurons (Owman and Sjostrand 1965) while the blood vessels of the uterus are supplied by ordinary long adrenergic neurons from the inferior mesenteric ganglia and other abdominal ganglia (Kanerva 1972 Kanerva *et al* 1972 b) Both the vascular and the extravascular cholinergic nerves have their origin in the paracervical ganglion (Kanerva *et al* 1972 b) The short adrenergic neurons differ both functionally and anatomically from the long adrenergic neurons (Sjoberg 1967 Swedin 1971) Pregnancy and administration of estrogen increase the amount of noradrenaline and the density of the adrenergic nerve net in the uterus of the rabbit but not in tissues innervated by long adrenergic neurons (Sjoberg 1967) The AChE activity in nerve fibres of the uterus has also been reported to fluctuate during the estrous cycle (Adham and Schenk 1969)

Large species variation exist in the anatomical and functional innervation of the female genital tract (Sjoberg 1967 Marshall 1970) Therefore a study was made on the effect of estrous cycle pregnancy and estrogen treatment on the innervation patterns of the rat uterus An abstract has previously been published (Kanerva and Lietzen 1971) Since then a paper has appeared dealing with the effect of pregnancy on the cholinergic fibres of the rat uterus (Paraventi and da Silva Sasso 1970)

References

BENNETT M. R. G BURNSTOCK and M. E HOLMAN Transmission from intramural inhibitory nerves to the smooth muscle of the guinea pig taenia coli. *J Physiol* (Lond.) 1966 *182* 541—558

BIBER, B O LUNDGREN and J SVANVIK Studies on the intestinal vasodilatation observed after mechanical stimulation of the mucosa of the gut. *Acta physiol. scand* 1971 *82* 177—190

BULBRING E H KURIYAMA and T TOMITA Discrimination between the "inhibitory potential" and electrotonic hyperpolarization in the smooth muscle *J Physiol* (Lond.) 1965 *181* 8—10 P

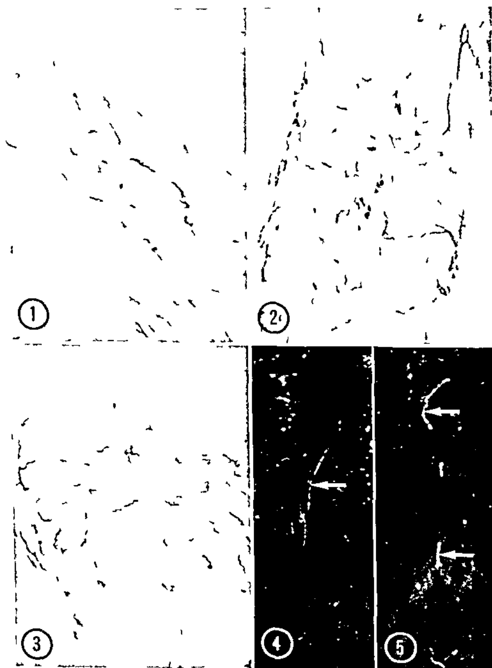
BULBRING E and T TOMITA Properties of the inhibitory potential of smooth muscle as observed in the response to field stimulation of the guinea pig taenia coli. *J Physiol.* (Lond.) 1967 *189* 299—315

CAMPBELL G Nerve-mediated excitation of the taenia of the guinea pig caecum. *J Physiol* (Lond.) 1966 *185* 148—159

GERSHON M D Effects of tetrodotoxin on innervated smooth muscle preparations. *Brit J Pharmacol.* 1967 *29* 259—279

HOLMAN M E. and J R. HUGHES Inhibition of intestinal smooth muscle *Aus J exp Biol. med Sci* 1965 *43* 277—290

PATON W D M The response of the guinea pig ileum to electrical stimulation by coaxial electrodes. *J Physiol* (Lond) 1955 *127* 40—41 P



See opposite page

the individual fibres was stronger After 3 weeks of pregnancy the size of the uterus still increased but the density and intensity of the AChE reaction of the nerve fibres decreased (Fig 3)

Changes after estrogen treatment The estrogen treatment caused the same effects as the 2 first weeks of pregnancy the uterus incrased in size and the fluorescence intensity in the few nonvascular fibres increased No changes was noticed in the vascular adrenergic nerves The AChE activity of the nerve fibres increased after one and further after 2 weeks of estrogen administration After 3 or 4 weeks of estrogen treatment no further increase in the fluorescence or AChE activity was noticed

The distribution of non specific cholinesterases (ns ChE) followed the distribution of AChE activity but was also intense around the glands and sensory endings in the perimetrial coat of the uterus The fluctuations in AChE activity and distribution of AChE positive fibres were accompanied by similar fluctuations in ns ChEs

Discussion

The density of extravascular adrenergic nerves in the myometrium of the rat is small compared to other mammals (see Sjoberg 1967) and has even been denied (Norberg and Fredricsson 1966) The effect of pregnancy and estrogen treatment on the adrenergic fibres of the uterus proved identical to the results obtained from rabbit (Sjoberg 1967)

It was previously noticed that the cell bodies of the short adrenergic neurons which are situated in the paracervical ganglion are affected by hormonal changes of the animal the fluorescence intensity of the adrenergic ganglion cells increased after two weeks of pregnancy (Kanerva *et al* 1972 a) This and the present results could be due to (1) an increased uptake of catecholamines (2) an increased catecholamine synthesis and (3) a lowered rate of release or axonal transport of catecholamines in the soma of the neuron Signs of an increased synthetic activity has been reported in the ganglion cells of the paracervical ganglion during pregnancy (Kennedy 1929) It was also recently proposed that the small intensely fluorescent (SIF) cells of the paracervical ganglion could be the source of catecholamines and thus explain the fluctuations in catecholamine concentrations of the uterus during the estrous cycle pregnancy and after hormone treatment (Kanerva and Teravainen 1972)

In previous studies it was observed that the concentration of AChE in the nerve fibres gradually decreased as pregnancy advanced (Makela and Gronroos 1959 Paraventi and Da Silva Sasso 1970) They both studied the AChE activity only after about 1 and 3 weeks of pregnancy Thus they did not notice the increase in the activity of cholinesterases after 2 weeks of pregnancy This is still compatible with the assumption that estrogen increases the activity of cholinesterases (Moricons *et al* 1966) while progestins decrease the AChE activity (Dumont *et al* 1960)

It has also been shown that estrogens increase the amount of catecholamines in the short adrenergic nerves innervating the female genital organs while progesterone

has an opposite effect (Falck *et al* 1969) This was specific for the short adrenergic neurons The estrogen effect on the AChE activity seems not to be specific for the uterus since increased cholinesterase activity has also been noticed in the lungs after estrogen treatment (Abdul Karim *et al* 1971)

The significance of the autonomic innervation of the uterus is not well known (Marshall 1970), especially not the changes observed in the nerves during pregnancy and after hormone treatment It seems probable that complex endocrine interactions affect not only the muscle cells but also the nerves of the uterus For a better understanding of these changes a larger project on the ultrastructure of the autonomic nerves in the female genital tract of normal pregnant and hormone treated animals is in progress in our laboratory (Hervonen and Kanerva 1972 a b Hervonen *et al* 1972 a b)

References

ABDUL KARIM R W M DRUCKER and R D JACOBS The influence of estradiol 17 β on cholinesterase activity in the lung *Amer J Obstet Gynec* 1970 *108* 1098—1101

ADHAM N and E A SCHENK Autonomic innervation of the rat vagina cervix and uterus and its cyclic variation *Amer J Obstet Gynec* 1969 *104* 508—516

ERANKO O The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence *J Roy micr Soc* 1967 *87* 259—276

ERANKO O and L ERANKO Small intensely fluorescent granule containing cells in the sympathetic ganglion of the rat *Progr Brain Res* 1971 *34* 39—51

DUMONT L A GERMAIN and M BAZIN Action de la progestérone sur l activite cholinestera s que du myometre du rat. *C R Soc Biol* (Paris) 1960 *154* 2329—2332

FALCK B CH OWMAN E ROSENGREN and N O SJOBERG Reduction of the estrogen induced increase in transmitter level of the short adrenergic neurons innervating the uterus *Endocrinology* 1969 *84* 958—959

GOMORI G *Microscopic Histochemistry* Chicago University Press Chicago 1952

HERVONEN A and L KANERVA Adrenergic and nonadrenerg c axons of the rabb t myometrium and oviduct *Acta physiol scand* 1972 a. *85* 139—141

HERVONEN A and L KANERVA Neuro-endocrine link in the regulation of myometrial activity In *The 4th International Congress of Histochemistry and Cytochemistry* Kyoto 1972 b

HERVONEN A L KANERVA R. LIETZEN and S PARTANEN Ultra tructural changes induced by estrogen in the adrenergic nerves of the rabbit myometrium *Acta physiol scand* 1972 a *85* 283—285

HERVONEN A L KANERVA and L RECHARDT Local zation of catecholamines and acetyl cholinesterase in the terminal nerve fibres of the rabbit myometrium *Histochemie* 197? b In press

KANERVA L The postnatal development of monoamines and cholinesterases in the paracervical ganglion of the rat uterus *Progr Bra n Res* 1971 *34* 433—444

KANERVA L Development, histochemi try and connections of the paracervical (F anken hauser) ganglion of the rat uterus. A light and electron microscopic tudy *A ta inst anat* (Helsinki) 1972 Suppl 2 1—31

fibres of the uterus during the estrous cycle during pregnancy and after estrogen treatment

KANERVA L and R. LIETZEN Histochem cal changes in the adrenergic and cholinergic nerve fibres of the uterus during the estrous cycle during pregnancy and aft estrogen treatment in the mouse and in the rat *Scand J clin Lab Invest* 1971 Suppl 116 65

KANER A L R LIETZEN and H TERAVAINEN Catecholamines and cholinesterases in the para cervical (Frankenhauser) ganglion of normal and p egnant rats *Acta physiol cand* 197? a *86* 271—277

KANERVA L. T MUSTONEN and H TERAVAINEN Histochemical tudies on the uterine inner vation after neure tomies *Acta phys ol scand* 1972 b *86* 359—365

KANERVA L and H TERAVAINEN Electron microscopy of the paracervical (Frankenhauser) ganglion of the adult rat *Z Zellforsch* 1972 *129* 161—177

KENNEDY W P The ganglion cervicalia uteri and the oestrus hormone *Edinb med J* *36* 75—88

KOELLE G B The elimination of enzymatic diffusion artefacts in the histochemical localization of cholinesterases and a survey of their cellular distribution *J Pharmacol exp Ther* 1951 *103* 153—171

LONG J A and H M EVANS The oestrus cycle in the rat and its associated phenomena *Mem Univ Calif* 1922 *6* 1—148

MARSHALL J M Adrenergic innervation of the female reproductive tract anatomy physiology and pharmacology *Ergebn Physiol* 1970 *62* 6—67

MORICONI A A DEBENEDETTI and A LUCARONI L attività colinesterasica nell utero del ratto Azione dell estradiolo e del progresterone *Boll Soc ital Biol sper* 1966 *42* 970—972

MAKELA S and M GRONROOS The cholinesterase activity of the uterus and Frankenhausers ganglion *Ann Med exp Fenn* 1959 *37* 407—413

NORBERG K. A and B FREDRICSSON Cellular distribution of monoamines in the uterine and tubal walls of the rat *Ac a physiol scand* 1966 *68* Suppl 277 149

OWMAN CH and N O SJOSTRAND Short adrenergic neurons and catecholamine containing cells in vas deferens and accessory male genital glands of different mammals *Z Zellforsch* 1965 *66* 300—320

PARAVENTI H and W DA SILVA SASSO Histochemical behaviour of the acetylcholin sterase in the myometrium of the albino rats during estrus diestrus pregnancy and parturition *Acta histochem* (Jena) 1970 *38* 70—73

SJOBERG N O The adrenergic transmitter of the female reproductive tract Distribution and functional changes *Acta physiol scand* 1967 Suppl 305 5—26

SWEDIN G Studies on neurotransmission mechanisms in the rat and guinea pig vas deferens *Acta physiol scand* 1971 Suppl 369

Acta physiol scand 1973 87 289—295

From the Department of Biochemistry of the Nobel Medical Institute Karolinska Institutet Stockholm and the Institute of Preventive Medicine University of Napoli Italy*

On the Regional Metabolism of Beef Heart Ventricles

By

B Tota**

Received 16 March 1972

Abstract

Tota B *On the regional metabolism of beef heart ventricles* Acta physiol scand 1973 87 289—295

The concentrations of some oligoelements (iron copper zinc) and electrolytes (calcium magnesium potassium) and the activity of the succinate-cytochrome c reductase system were determined in various regions of the ventricle of beef heart In the subepicardial layers of the ventricles and in the upper part of the septum a higher content of iron and copper and a greater succinate-cytochrome c reductase activity were observed These findings were consistent with a decreasing oxidative metabolic gradient both from the epicardium to the endocardium in the free ventricular walls and from the upper to the lower part of the septum A higher content of potassium was demonstrated in the subendocardium of the ventricles Significant differences in the concentrations of zinc magnesium and potassium were found between the right and left papillary muscles

A gradient of intramyocardial pressure from epicardium to endocardium in the ventricular wall of mammalian heart has been recognized since the time of the pioneering investigations of Anrep and his associates (1928 1931 1933) and those of Johnson and DiPalma (1939) The occurrence of such a gradient which subsequently has been confirmed by several authors employing more sophisticated methods (Gregg and Eckstein 1941 Laszt and Muller 1958 Kirk and Honig 1962 1964 a) has stimulated in recent years a more detailed study of some physico chemical and biophysical gradients in the ventricular wall of the heart Particularly interesting is the significant reduction of oxygen tension (Moss 1966 1968) and of blood supply (Kirk and Honig 1964 b Brandi Fam and McGregor 1966 Moir and De Bra 1965 1967 Kober and Kaltembach 1971) reported in the subendo cardium since such reductions have been thought to be the cause of the greater sensitivity of this layer to hypoxia and stress especially in the left ventricle (as for instance under pressure overload)

* This work has been carried out partly at the Physiological Department of the Zoological Station of Napoli Italy

** Present address Istituto di Medicina Preventiva, Universita di Napoli Policlinico—80138 Napoli Italy

Fig 1 The myocardial layers (in black) were prepared as it is shown in *a* for the determination of the oligoelements and electrolytes and as it is shown in *b* for the determination of the succinate cytochrome c reductase activity

On the other hand the experimental biochemical findings which correlate the metabolic adaptations of the various layers of the ventricles with the described gradients are so far very few

We have therefore taken up a study of the biochemical heterogeneity of mammalian ventricular myocardium and the purpose of this paper was to investigate whether there were significant metabolic differences in various myocardial regions of the beef heart ventricles

Since the inorganic metabolism has never been systematically studied in the ventricular myocardium a portion of this work was devoted to obtaining biochemical information on the distribution of some oligoelements (iron copper zinc) —which are known to be closely correlated with specific enzymatic systems (Table I) —and of some electrolytes (calcium magnesium potassium) the remaining part of this study was designed to give information on some aspects of the oxidative metabolism and therefore the activity of succinate cytochrome c reductase which is an integral component of the mitochondrial respiratory chain was determined

Materials and methods

The hearts were excised from cows between 19 and 24 months of age immediately after the animals were killed and atria great vessels fat and ligaments were trimmed away The ventricles were cut open and rinsed of blood in ice-cold physiological solution

Oligoelements and Electrolytes Multielement determinations b atomic absorption spectrophotometry were carried out Well aware of the main problems of contamination in trace analysis during all the steps of the experiments (sampling packaging and anal tical procedure) we followed the specific procedures of Bowen (1966) and Scott (1970)

From 3 hearts the subepicardial and subendocardial layers (each one 3—4 mm thick) were separated from 3 different levels of the free right and left ventricular walls the upper and lower parts of the interventricular septum and the right and left papillary muscles (see Fig 1) The muscle samples cleaned of visible fat and connective tissue and prepared as a fine mince were divided into 2 parts the main part (I) being intended for water fat oligoelements and electrolytes determination and the other part (II) for muscular protein determination

The samples from part I (200—400 mg each) were weighed dried at 90 C for 2 h and afterwards reweighed in the presence of phosphorus pentoxide Water content was obtained b difference between wet and dry weights

Fat extraction by petroleum ether was then performed according to Bergstrom (1962) The fat free solid (FFS) was used as a reference base for oligoelements and electrolytes The metals were extracted by nitric acid 65 (10 ml/100 mg of wet weight) the tissue samples being completely dissolved in 2 days

The readings were carried out by a Densatomic Optica Atomic Absorption Spectrophotometer connected with a recorder using a gas mixture of acetylene and air Analyses were determined in duplicate in each case

TABLE I Myocardial metalloproteins containing Iron Copper or Zinc

Iron	Cytoplasmic dehydrogenases Mitochondrial dehydrogenases (Catalase Myoglobin) Non heme iron of the respiratory chain Cytochromes b c c
Copper	Cytochrome oxidase
Zinc	(Lactate Dehydrogenase *Glutamate Dehydrogenase*) Malate Dehydrogenase

TABLE II Distribution of Iron Copper and Zinc in Beef Heart Ventricles

	Feγ/100 mg F F S	M P	Cuγ/100 mg F F FS	M P	Zn /100 mg F F S	M P
R V Subepicardium	42 46±1 8	30 39±2 8	6 23±0 3	4 47±0 9	30 77±7 5	22 18±5 9
Subendocardium	40 26±2 1	23 26±1 7	3 91±0 1	2 10±0 4	27 22±7 4	16 20±2 9
n = 18						
L V Subepicardium	53 24±1 9	39 86±1 9	6 83±0 2	4 95±0 6	29.29±5 7	23 26±5 0
Subendocardium	47 08±0 6	35 65±0 8	4 10±0 1	2 52±0 3	27 69±5 9	20 69±4 7
n = 18						
R Papillary muscles	42 04±2 0		7 82±0 8		33 60±2 1	
L Papillary muscles	46 16±2 2		6 03±1 1		25 79±2 5	
n × 12						
Upper Interv Septum	40 2 ±2 1		7 80±2 0		30 87±2 3	
Lower Interv Septum	35 0±1 8		3 58±1 3		19 44±2 7	

F F S = fat free solid M P = muscle proteins R = Right L = Left V = Ventricle Interv = = Interventricular

TABLE III Distribution of Calcium Magnesium and Potassium in Beef Heart Ventricles

	Ca γ F F S	Mg γ F S S	K mg F F S
R V Subepicardium	52 46±7 9	76 40±3 5	3 10±0 2
Subendocardium	47 46±7 4	73 35±2 6	4 01±0 3
n = 18			
L V Subepicardium	39 03±2 3	90 88±4 3	4 21±0 2
Subendocardium	39 25±5 4	93 33±6 1	4 84±0 1
n = 18			
R Papillary Muscles	35 01±2 1	88 98±2 1	4 03±0 2
L Papillary Muscles	33 98±2 0	94 51±1 0	4 72±0 3
n = 12			
Upper Interv Septum	30 07±2 3	94 34±2 5	4 86±0 2
Lower Interv Septum	32 95±2 8	93 43±3 0	4 19±0 2
n = 12			

F F S = fat free solid R = Right L = Left V = Ventricle Interv = Interventricular

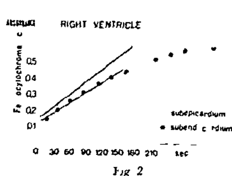


Fig 2

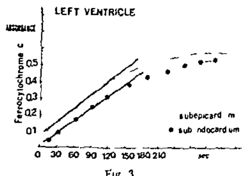


Fig 3

Fig 2 Activity of the succinate-cytochrome c reductase system The concentration of ferrocytochrome c (*mg/ml*) *is plotted* versus time (sec) Composition of the reaction mixture Phosphate buffer (*pH 7 4*) *0 1 M ETP 0 15 mg proteins* KCN 5 x 10 3 mM Cytochrome c (Horse Heart—Type III Sigma) 2 mg Succinate 10 0 mM T = 37 C

Fig 3 Same conditions as in Fig 2

All standards and solutions were prepared with deionized distilled water As far as analyses of iron copper and zinc are concerned there are no significant interferences (Elwell and Gidley 1966) and therefore the preparation of the standards met no special problems For calcium magnesium and potassium however several possible types of interference must be taken into account the interference occurring in calcium analysis was almost completely overcome by employing a diluting fluid containing strontium chloride (1 %) while for magnesium and potassium the method of Beroniade Bergstrom and Hultman (1968) was followed

The absorption values were statistically evaluated according to the method of linear regression

Muscle protein determination The muscle sample (part II) intended for muscle protein (MP) determination was treated as described by Bergstrom (1962) The non-collagen proteins which are directly correlated with the cell mass independent of the connective tissue content (Lilienthal *et al* 1950) were used as another basis of reference for the oligoelements

Determination of the succinate cytochrome c reductase activity From the free right and left ventricular walls of 2 hearts the complete subepicardial and subendocardial layers (each one 3—4 mm thick) and the complete transverse section (2 5 cm long) of the upper and lower parts of the septum were prepared (Fig 1) An electron transport particle (ETP) preparation was obtained from each of these parts by the method of Keilin and Hartree (1939) modified as described by Tota (1970) Protein concentrations in each of the twelve preparations calculated from micro-Kjeldahl analyses (Niederl and Niederl 1942) using 6 25as the conversion factor were adjusted to 25 mg/ml with an appropriate amount of 0 1 M phosphate buffer pH 7 4 Subsequently 2 ml aliquote of these suspensions were dispensed into ampoules frozen in an acetone/solid carbon dioxide bath and stored at −80 C In this way the succinate cytochrome c reductase activity remains unchanged for many months (Tota 1970) All the assays were run within one month For determining the enzymatic activity the method described by Paleus *et al* (1969) was employed

Results

The results are summarized in Table II and III (metals) and in Fig 2 3 and 4 (succinate cytochrome c reductase activity) from which it follows

I In the left ventricle there are higher concentrations of iron and copper in the subepicardium than in the subendocardium when the basis of reference is expressed either as FFS or as muscle proteins (MP) In the right ventricle the same situation is found for copper (the transmural gradient being however of smaller magnitude

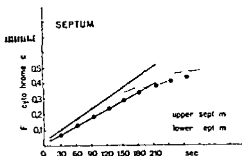


Fig 4 Same conditions as in Fig 2

than that of the left ventricle) but the iron concentration appears to be higher in the subepicardium only when expressed in terms of M P The right papillary muscles have a higher concentration of zinc than do the left papillary muscles

A longitudinal gradient occurs in the interventricular septum for all the oligo-elements analysed the upper region being richer in oligoelements than the lower one

2 In both ventricles the subendocardial layers have a higher content of potassium than do the outer subepicardial layers No significant differences have been observed for calcium and magnesium concentrations

The left papillary muscles are richer in potassium and magnesium than the right papillary muscles A longitudinal gradient of potassium similar to that present for iron copper zinc occurs in the septum

3 Fig 2 3 and 4 represent the activity of ETP preparations which is expressed as the quantity of cytochrome c reduced by succinate as a function of reaction time The figures demonstrate a) in both hearts the ETP prepared from subepicardial layers had an enzymatic activity higher than that shown by the ETP prepared from subendocardial layers b) the difference between the enzymatic activity of the inner and outer layers is larger in the left ventricle than in the right c) the succinate cytochrome c reductase activity is higher in the upper part of the septum

Discussion

The complex biochemical heterogeneity of the ventricular myocardium is evident from these data The view of metabolic transmural gradients demonstrated by Jedeikin (1964) and by Kirk Honig and Myers (1967) is supported by our results as far as the beef heart is concerned In the subepicardium of both ventricles there is in fact a significant correlation between the higher concentrations of iron and copper and the greater activity of succinate-cytochrome c reductase system In the skeletal muscle and myocardium most of the copper is bound to the cytochrome oxidase molecule the terminal enzyme of the mitochondrial respiratory chain This has also been confirmed in beef heart by Wester (1965) In several investigations therefore the presence of copper has been demonstrated to be a good indicator of mitochondrial oxidative metabolism

ANREP G V and H HAUSLER The coronary circulation I The effect of changes of blood pressure and of the output of the heart *J Physiol* (Lond) 19?? 6? 3?—?
ANREP G V J C DAVIS and E VOLHARD The effect of the pulse pressure ... coronary blood flow *J Physiol* (Lond) 1931 *73* 405—4 6
ANREP G V and E SAAFELD The effect of the cardiac contraction upon the coronary ... *J Physiol* (Lond) 1933 *79* 317—331
BASILE C and B TOTA Distribuzione degli isoenzimi della lattico deidrogenasi ... parti del miocardio bovino *Boll Soc ital Cardiol* 1971 *16* ...
BERGSTROM J Muscle Electrolytes in Man *Scand J clin Lab Invest* ...
BERGSTRADE V J BERGSTROM and E HULTMAN Potassium and Magnesium ... needle biopsy specimens of human muscle tissue determined by atomic absorption spectrophotometry To be published
BOWEN H J M Trace Elements in Biochemistry Academic Press London ...
BRANDI G W M FAM and M McGREGOR Estimation of coronary flow ... injections of radioactive tracers *Circulation* 1966 34 Suppl 3 ...
DALLNER G Studies on the Structural and Enzymatic Organization of the Membranous Elements of Liver Microsomes M D Dissertation *Acta path microbiol scand* ... Suppl 166
ELWELL K and J A GIDLEY *Atomic Absorption Spectrophotometry* Pergamon Press ... 1966 p 89—95
ESTES E H M L ENTMAN H B DIXON and D B HACKEL The vascular supply of the left ventricular wall *Amer Heart J* 1966 *71* 58—67
GREGG D E and R W ECKSTEIN Measurements of intramyocardial pressure ... *Physiol* 1941 *132* 781—790
JEDEIKIN L A Regional distribution of Glycogen and Phosphorylase in the ventricles of the heart *Circulat Res* 1964 *16* 202—211
JOHNSON J R and J R DIPALMA Intramyocardial pressure and its relation to ... pressure. *Amer J Physiol* 1939 *125* 234—243
KEILIN D and E F HARTREE Cytochrome oxidase *Proc roy Soc* ...
KIRK E S and C R HONIG Physico-chemical gradients in the left ventricle ... *Proc* 1962 21 (2) 105
KIRK E S and C R HONIG An experimental and theoretical analysis of ... pressure *Amer J Physiol* 1964 207 (2) 361—367
KIRK E S and C R HONIG Nonuniform distribution of blood flow and gradients of oxygen tension within the heart *Amer J Physiol* 1964 207 661—...
KIRK E S C R HONIG and W W MYERS Transmural Distribution of ... *tension and Metabolism in Myocardium Mechanism and ... tion and Energetics of the Myocardium* Karger Basel 1967 ...
KOBER G and M KALTENBACH Regional Myocardial Blood Flow ... In *Coronary Heart Disease* G Thieme Verlag Stuttgart 1971 ...
LASZT L and A MULLER Der myocardial Druck *Helv physiol pharmacol Acta* ... 88—106
LILIENTHAL J L K L ZIERLER B P FOLK R BUKA and M J RILEY ... and system of analysis of muscle constituents *J biol Chem* ...
MOIR T W and D W DEBRA Measurements of the endocardial ... coronary blood flow by Rb^{86} Chloride *Amer Heart J* ...
MOIR T W and D W DEBRA Effect of left ventricular Hypertension ... active Drugs on the Myocardial Distribution of Coronary flow *Circulat Res* 1967 ... 65—74
MOSS A J Intramyocardial oxygen tension *Circulation* ...
MOSS A J Intramyocardial oxygen tension *Cardiovasc Res* ...
NIEDERL J and U NIEDERL Micromethods of quantitative organic ... 1947 51
PALEUS S B TOTA E HULTIN and G LILJEQVIST ... Reductase Activity in the Keilin Hartree Beef Heart Preparation *Acta chem scand* ... 23 1—6
SCOTT R O Problems in Trace element Analysis In *Trace Elements ...* Livingstone Edinburgh and London 1971 497—501
TOTA B Proprieta e determinazione della attivita ... cardiomuscolare di Keilin Hartree modificato *Boll Soc ital Biol sper* ...
WESTER P O Concentration of 17 elements in subcellular fractions of ... determined by neutron activation analysis *Biochim biophys Acta* (Amst) ... 268—283

Acta physiol scand 1973 87 296—306

From the University Institute for experimental Medicine Copenhagen Denmark

A Study of the Effects of Some Chemically Welldefined Lissamine Greens on Tubular and Overall Renal Function in the Rat (with an evaluation of the validity of lissamine green transit time measurements in studies of tubular function)

By

MOGENS ELMER and PAUL P LEYSSAC

Received 24 May 1972

Abstract

ELMER M and P P LEYSSAC *A study of the effects of some chemically welldefined lissamine greens on tubular and overall renal function in the rat (with an evaluation of the validity of lissamine green transit time measurements in studies of tubular function)* Acta physiol scand 1973 87 296—306

Chemically different Lissamine greens were investigated for their immediate and late effects on renal functional parameters. The data indicate (1) that there was no consistent change in transit times (TT) in series of up to four repeated single injections with different LG completed within 8 min, (2) proximal and distal intratubular pressure responded to a single i.v. injection of LG with two distinct pressure increments similar for all dyes tested. The first increase (prox. 1—3 mm Hg, dist. 1—2 mm Hg) coincided with a blood pressure response, the second increase (prox. 1—2 mm Hg, dist. 0.5—1.5 mm Hg) started when LG appeared in distal convolutions. The changes in flow velocity resulting from such pressure changes would not cause any significant error in the measured TT, (3) the late effects of LG were an increase in overall reabsorption capacity (predominantly in the loop of Henle), a parallel increase in glomerular filtration rate, and an increase in urine flow and osmotic excretion rate. Proximal reabsorption rate was unaffected. No difference in response was observed between different LG. Neither GFR nor urine flow changed significantly in control experiments in which a similar osmotic activity of mannitol was injected. It is concluded that TT measurements with LG may provide valid information on tubular function, but the late effects of LG would limit its use to a single measurement at the end of an experiment.

Key words: Transit time, tubular function, tubular hydrodynamics, natriuresis

---

Lissamine green (LG) has been widely used in studies of tubular function over the last decade being a simple means for visualizing the tubular fluid flow following intravenous or intraluminal administration.

Recent in vitro studies on the effect of LG on transcellular fluid and/or sodium transport have demonstrated that under these conditions the dyes may inhibit the absorptive process to varying degrees both in the frog skin and the rabbit gallbladder

(Dorge and Nagel 1969 Christensen and Frederiksen 1972) Further Roch Ramel *et al* (1970) and Heller (1971) have reported a natriuretic and diuretic effect of repeated intravenous injections of LG

It was therefore motivated to analyze in some further detail the immediate and delayed renal effects of some Lissamine greens The results obtained provide a more extensive basis for evluation of the limitations and field of applicability of LG in future renal research

Methods

White male SPF rats weighing 240—300 g allowed free access to food and water prior to the experiment were anesthetized with intraperitoneal injections of sodium amobarbital (sodium Amytal) 15 mg/100 g body weight (BW) Anesthesia was maintained when necessary with intermittent intraperitoneal injections of sodium Amytal The rats were placed on a heated operating table Body temperature was maintained at about 37 C by a rectal thermometer controlling the heat regulation The rats were prepared for micropuncture and occlusion time measurements as previously described (Leyssac 1963 1964) Polyethylene catheters were inserted into the left jugular vein for infusion of inulin and injections of Lissamine green into the right carotid artery for blood collection and continuous measurement of systemic arterial pressure into the left ureter for urine collection The surface of the left kidney with its capsule intact was illuminated with fiber optics and irrigated with Ringer solution preheated to 37 C

The rats received priming doses of 15—20 mg of inulin in 10 % solution followed by continuous infusions of 1 % inulin in 0 9 % saline at a rate of 0 15 mg/min After an equilibration period of about 45 min 2 to 4 serial urine samples were collected within 15—30 min for clearance determinations Arterial blood samples (about 200 µl) were collected before and after each clearance period urine samples were collected directly into calibrated polyethylene tubing (PE 50) and the volume excreted determined from the length of the urine column Inulin in plasma and urine were measured by the diphenylamine method of Bojesen (1952) modified for microanalysis

Plasma and urine osmolality was measured cryoscopically by the ultra micromethod of Ramsay and Brown (1955)

Systemic arterial pressure (BP) was monitored continuously throughout all experiments with a Hansen capacitance pressure transducer (S a W) connected to a Varian or Servogor recorder Intratubular pressures (ITP) were measured by the Landis method essentially as described by Cottschalk and Mylle (1956) The pressure to the micropipette (outer tip diameter 8 µm) was adjusted by means of a mercury levelling bulb and transferred to the pipette and a Hansen capacitance pressure transducer through stiff water filled tubings With this arrangement 5—10 measurements of intratubular pressure could be made per minute even when the pressure was rapidly changing

Proximal luminal occlusion time (OT) was measured immediately after the last urine collection in clearance experiments as described previously (Leyssac 1964) Proximal transit time (TT_P) was measured in the modification of Steinhausen s LG method (1963) as described by Gertz *et al* (1965) Paired measurements of OT and TT by two investigators agree within 3 s and 2 s respectively (Bojesen and Leyssac 1969) Transit time through the loop of Henle (TT_H) was measured as the time interval between dye appearance in the cortical capillaries and the appearance in the distal convolutions subtracted by TT_P For transit time measurements 75—100 µl 5 % LG in 0 9 % saline titrated with NaOH to pH 7 4 were rapidly injected i v

Four chemically different Lissamine greens were tested 1) an unspecified LG-dye mixture 2) the triphenylmethane derivate LG 240 SF (MW 792 8) 3) and the two diphenyl naphtylmethane derivatives LG 162 B (MW 576 6) and LG 280 V (MW 616 7)* For chemical formulars of the dyes tested *cf* paper by Christensen and Frederiksen (1972)

Experimental protocols

Group A In 11 rats proximal (TT_P) and Henle transit time (TT_H) were measured after each of several repeated i v single injections at 2 min intervals in each experiment 2 to 4 of the different LG were tested With the exception of protocol no 868 and 876 these experiments were carried out at the end of a clearance experiment

* All LG dyes tested were obtained from E Gurr Ltd (London)

Group B In 49 expts on 17 rats changes in proximal or distal intratubular pressure with time following a single i v injection of 100 μl 5 LG or 0 9 % NaCl were recorded by rapidly consecutive timed pressure measurements at the same puncture site ITP were measured first in a control period lasting about 2 min when the pressure was stable within the normal range of values a rapid injection of LG or saline was given pressure measurements were continued during the injection and in the following period for up to 10 min after the injection or in ome experiments only until the pressure had stabilized 22 expts were carried out with LG 240 SF 15 expts with LG 280 V 1 exp with the unspecified LG mixture and 11 expts with saline

Group C Clearance experiments were carried out in 12 rats receiving LG and in 11 control rats receiving the same osmotic activity in the form of mannitol Proximal and distal intra tubular pressures were measured in several (1—8) convolutions during each urine collection period The mean pressure for the clearance period is given After collecting the first blood sample a control period was started which included measurement of the OT immediately after the last urine collection 1—3 min after the OT measurement when systemic arterial pressure as well as urine flow had returned to control levels a single injection of 75—100 μl (5 % in 0 9 saline) of one of the Lissamine greens tested was given rapidly i v and transit times were measured 100 μl 2 9 % mannitol in 0 9 % saline was given in control experiments A second blood sample was collected afterwards

Urine flow usually increased within few minutes after dye appearance in the uretheral catheter and stabilized at a new level within 10—15 min In control experiments only a minor brief and inconsistent rise in urine flow was seen As soon as urine flow appeared stable a second (experimental) clearance period was started At that time urinary LG concentration was about 0 05 % (0 6—0 8 mM) as estimated colorimetrically At the end of the last urine collection OT and TT were measured again using the same LG as in the control period After collecting a third blood sample the left kidney was removed drained and weighed

LG 280 V was tested in 5 expts LG 240 SF in 6 expts and the unspecified LG mixture in 1 expt

Calculations Inulin clearance was calculated from the urine/plasma inulin concentration ratio (U/P_{IN}) and the volume of urine collected per minute (V_U) divided by the kidney weight (KW) by means of the conventional expression

$$Cl_{IN} = (U/P_{IN}) \cdot (V_U/KW) \text{ ml min}^{-1} \text{ g KW}^{-1} \quad (1)$$

End proximal tubular fluid/plasma inulin ration (TF/P_{IN}) was calculated using the expression derived by Bojesen and Leyssac (1969)

$$\ln (TF/P_{IN}) = TT_P/OT \quad (2)$$

The fraction of filtrate reabsorbed by the end of the proximal convolutions was estimated from the calculated TF/P_{IN} according to the expression

$$\text{reabsorbed fraction} = 1 - (P/TF_{IN}) \quad (3)$$

The absolute rate of proximal reabsorption was calculated from the Cl_{IN} and the fraction reabsorbed by the proximal convolutions

The rate of reabsorption in nephron segments distal to the proximal convolution (lower nephron segment including pars recta of the proximal tubule loop of Henle distal tubule and collecting tubule and duct) was calculated as the difference between overall tubular reabsorption rate and reabsorption rate by the proximal convolutions

Results

Group A The results of transit time measurements following repeated single injections at short time intervals of the various LG dyes tested are presented in Fig 1 It is apparent first that there was no consistent change either in TT_I or TT_{II} in series of up to four repeated measurements completed within 8 min The difference observed between the single measurements in a series was within the difference observed between individual nephron groups in a field of vision Thus the differences between transit times obtained in a series were not significant Secondly the data show that any consistent difference between transit times (TT_I as well as TT_{II}) obtained with four chemically different LG dyes could not be observed

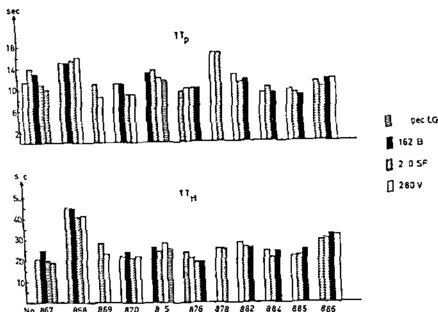


Fig 1 Transit times following repeated single injections of various LG dyes The numbers given below the bars refer to protocol numbers of the individual rats TT_P proximal transit time TT_H transit time through the loop of Henle

Group B The effect of a single i v injection of 100 μl 5 % LG (pH 7 4) on arterial blood pressure and proximal intratubular pressure showed a characteristic and consistent pattern similar for all LG dyes tested Recordings from a representative experiment are shown in Fig 2

The LG effect on arterial pressure was biphasic immediately after the injection pressure increased 5—10 mm Hg for about 2—6 s the pressure then dropped to 5—10 mm Hg below the initial level and returned to control value within 5—15 s Arterial pressure then remained stable The proximal intratubular pressure responded to a single LG injection with two distinct pressure increments (an M shaped pressure time curve) The initial pressure increase started immediately after the dye injection the pressure increase reached a maximum of 1—3 mm Hg and then returned to control baseline within 10—20 s the first increase thus coincided with the BP response The second increase in proximal ITP started either immediately before or at the same time as LG appeared in the distal tubule *i e* usually 30—40 s after the injection when the BP had returned to a stable control value The second pressure increase was usually slightly smaller than the first peak (1 53 ± 0 20 (S E) mm Hg) and lasted for a longer but quite variable time (30—120 s) It was statistically significant ($p < 0\,001$)

The distal intratubular pressure change following a single LG injection was quite similar to that recorded in proximal convolutions only the absolute pressure change was smaller The initial increment was 1—2 mm Hg and started immediately afte

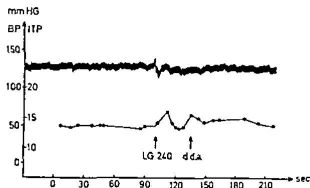


Fig 2 Characteristic pressure pattern in a surface proximal tubule (ITP) and arterial blood pressure (BP) following a single injection of LG dda denotes distal dye appearance

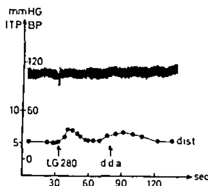


Fig 3

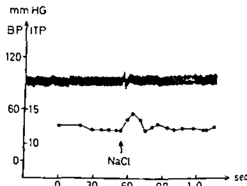


Fig 4

Fig 3 Characteristice pressure pattern in a surface distal tubule (ITP) and arterial blood pressure (BP) following a single injection of LG dda distal dye appearance

Fig 4 Characteristic pressure pattern in a surface proximal tubule (ITP) and arterial blood pressure (BP) following a single injection of 0 9 saline

injection the second increase in distal ITP was smaller than the first one (1 07 ± 0 24 (S E) mm Hg) and also started a few seconds before LG appeared in distal convolutions (Fig 3) It was statistically significant ($p < 0\ 01$) The effect of a single i v injection of an equal volume of isotonic saline on BP and proximal ITP differed clearly from that observed after LG Recordings from a typical experiment are shown in Fig 4 The BP response was an immediate mono or biphasic increase of 5—10 mm Hg of a few seconds duration ITP responded only with an initial brief elevation of 0 5—2 5 mm Hg (usually about 1 mm Hg) immediately following the injection A second significant pressure increase characteristic of the LG re ponse was not observed following saline injection a second increase was observed only in 3 of 8 expts and averaged 0 16 ± 0 09 (S E) mm Hg which is statistically insignificant ($0\ 02 > p > 0\ 1$) Thus the secondary pressure response in proximal and distal tubules following LG differed significantly from that observed after saline ($p < 0\ 001$ and $p < 0\ 00$ respectively)

TABLE I A summary of the effect of a single injection of LG on several functional parameters in clearance experiments OT proximal luminal occlusion time TTp proximal transit time corr OT/TT occlusion time/transit time ratio OT corrected for 12 % reflux (Bojesen and Leyssac 1969) TF/P$_{IN}$ end proximal tubular fluid/plasma inulin ratio Changes from control to experimental periods are presented as mean values ± 1 S E

p. to	LG spe-	clea anc Inul n mLm n⁻¹ g KW⁻¹		ι e flow µLm ⁻¹ g KW⁻¹		osm. r t µosmm ⁻¹ g KW		OT ec		TTp c		o OT/TT		T/P	
		cont	p	o t	p.	o t	p	cont	p	cont	e p.	ont	p	ont	e p.
878	80	096	10	190	433	330	768	210	225	65	165	112	120	2	
879	unsp.	140	182	340	380			1 0	170	100	120	150	125	195	3
882	80	058	111	279	269	506	498	145	150	140	1 5	091	106	30ι	8
884	80	213	359	3 36	492	629	87	120	130	100	0	106	12	2	
885	280	182	208	340	790	670	1105	12.5	135	110	95	100	125	2 2	2
886	240	115	162	251	435			26.0	22.5	210	110	109	180	250	1
887	240	096	143	159	338	280	5.42	30.0	230	110	120	240	169	15	18
888	40	093	105	169	234	322	424	14.0	230	13.0	140	092	14	2 P	0
893	240	138	179	388	515	360	890	14.0	14.0	14.0	100	0.88	127	3. 0	2
89	240	106	115	164	254	467	60	215	275	230	2 ω	08	1 0	3.	24e
895	2 0	128	097	293	212	4J7	282	125	190	95	110	116	157	23	1P8
896	280	118	115	239	314		620	130	145	95	130	1 0	3	23	b
m h q		030 013		127 039		2.06 0.81								-03- 0 4	
P		0.05		0.01		0.05		n. s						J	

TABLE II A summary of the effects of a single injectio nof LG on absolute rates of reabsorption, Henle loop transit time (TT$_H$) and intratubular pressures (ITP) in clearance experiments Changes from control to experimental periods are presented as mean values ± 1 S E

p o	LG ιec	s. t f eabs p l tion ml.m ¹g KW⁻¹		bs. of bs. ower ephr gm ml.mn ¹g KW⁻¹		TT$_H$ c		ITP$_p$ x mmHG		ITP$_{dist}$ mmHG	
		co t	p	t	p.	ont	p	t	p	o t	exp
878	280	057	0.59	0.39	045	29.0	25.0	12.2 0.1		6.3 0	
879	pec	068	100	0.72	082	260	200	131 03	115	65	
882	280	066	068	032	0.43	29.0	28.0	11.9 06	12.7 0.1		6.8 0.1
884	280	130	196	083	163	29.0	24 0	12 6 0.1	13.2 0.1	6.0 0.1	5.7
8d	28	1	115		0. 2	26.0	215	13.0			
P86	40	069	0.69	0 6	093	3.0	29.0	12.5 0.2	11.7 0.5	5.8 0.	5.0
887	240	033	064	0.63	079	3.0	25.0	11 5-0.	115 06	4.4 01	0
888	0	0.62	3	0.31	3 2	t.	1	12.6-0.3	13.2 1.	57 .1	58 0
8			0 6	0..	r 81	.0	4.0	12 8 0.3	12.7 1.9	0	57
6	0		060	032	0.6	.0	330	12 4±0.2	12.8 16	4.5 0.3	65
8	J	0	04E		051	195	2.0	130 0.3	130 04	60-0.3	59 01
896	80	06.	0 3	J.4	0.42	29.5	370	12 8.4	14.9 11	70 1.2	73 0.2
mean hang		0.09 0.07		+0.20 0.07		27 1.8		0.2 0.3		0.3 0.4	
P				0.02						s	

TABLE III A summary of control experiments The second period followed a single injection of mannitol (100 µl 29 %) The changes in clearance and urine flow from the first to the second period are given as mean values ± 1 S E

Exp No	clearance inulin ml min⁻¹ g KW⁻¹			urine flow µl min⁻¹ g KW⁻¹		
	period 1	period 2	change	period 1	period 2	change
1006	1 48	1 56	0 08	5 13	3 32	1 81
1007	0 82	0 78	0 04	2 27	2 79	0 .2
1008	1 68	1 30	0 38	10 80	11 30	0 0
1009	1 40	1 55	0 15	5 07	4 05	1 02
1010	1 03	1 40	0 37	5 92	5 66	0 26
1011	0 80	0 95	0 15	2 35	3 55	1 ?0
1012	1 04	1 17	0 13	4 48	5 74	1 26
1013	1 49	1 49	0 00	5 30	5 48	0 13
1014	1 70	1 37	0 33	5 7.	6 75	1 00
1015	1 84	1 22	0 62	8 91	8 91	0 00
1016	0 90	0 80	0 10	6 59	3 6	2 97
mean change ± E	0 054 0 057			0 134 0 499		
p	> 0 1 N			> 0 1 N S		

Group C The results of clearance experiments are given in Table I—III It appears that urine flow osmotic excretion rate and inulin clearance increased significantly and quite consistently in the second clearance period following the first single injection of LG *Urine osmolality* (U_{osm}) *remained unchanged* in this series of experiments In control experiments (Table III) in which an osmotic activity of mannitol equal to that of LG was given in a similar volume of saline inulin clearance and urine flow changed inconsistently The mean change in clearance was -0.054 ± 0.057 (S E) ml min⁻¹ g KW⁻¹ the change is not significant but differs significantly from the change observed after a single injection of LG ($p < 0.05$) Also the mean change in urine flow following a single injection of mannitol was insignificant

The changes in TT_I following LG did not show any consistent pattern TT_I increased slightly in 5 expts decreased in 6 expts and remained unchanged in one The mean change was insignificant OT remained unchanged or increased in most experiments so that a significant pattern appears in the TT/OT ratio—and thus in the calculated TF/P_{IN} ratio It is seen from Table I that the TF/P_{IN} ratio and thus the fraction of filtrate reabsorbed *proximally decreased* consistently in 9 out of 12 expts The absolute rate of proximal reabsorption *remained unchanged* (Table II)

The absolute rate of reabsorption in the lower nephron segments *i e* distal to the proximal convolutions increased significantly following exposure to LG (Table II),

and at the same time TT_{II} tended to be shorter than in the control period (in 9 out of 12 expts)

Proximal as well as distal ITP remained unchanged (Table II)

Any difference in response to LG 280 V and LG 240 SF could not be detected in any of the measured parameters

Discussion

One would predict that any major difference in the immediate tubular effect of chemically different Lissamine greens should be unmasked by consistent differences in the measured transit times in series of repeated single injections of the various dyes The results obtained here could not demonstrate any difference neither between TT_P nor TT_{II} with the four different dyes tested nor was there any consistent change in transit times following up to 4 repeated single injections of LG completed within 10 min This observation does not of course exclude that the injected dyes affected the tubular transport process during the measurement only it indicates that no matter whether or not LG affected the measured TT within the period of measurement the effect if any was equal for all the dyes tested

The lack of any consistent change in TT following repeated injections (group A) is apparently at variance with observations by Heller (1971) who found the proximal transit time to be on an average 1 7 s shorter following the fifth of repeated LG injections within 16 minutes The reason for this minor discrepancy is not quite clear Although it might be due to chemical differences in the dyes used a more likely explanation would seem to be the longer duration of Heller s experiments *i e* a result of a late secondary response to LG as observed in our clearance study (group C *cf* later in the discussion) Heller attributed tentatively the change in TT_P to inhibition of proximal reabsorption rate However TT_I depends on several other factors including the filtration pressure the proximal luminal diameter and the reabsorptive capacity and flow resistance in the more distal segments

More insight in the immediate effects of LG on tubular function may be obtained from the pressure measurements in group B The initial and immediate intratubular pressure increase of about 2 mm Hg following a single i v injection of LG coincided with a BP response and similar pressure changes arterially as well as intraluminally were observed following saline injection It is therefore most reasonable to interpret this tubular pressure elevation as a reflection of a change in filtration pressure resulting from the fluid volume expansion and the concomitant vascular reactions rather than any specific tubular effect of LG The pressure change is transmitted to the distal tubule and thus is consumed by the entire nephron A proximal pressure increment of about 2 mm Hg then amounts to about 15 % of the entire pressure drop along the nephron Evidence has been presented indicating that the tubular wall may resist minor pressure differences of a few mm Hg at least for a short period of time (Leyssac 1965) Thus it seems reasonable to assume that the luminal diameter is not significantly changed by the brief pressure increase of 2 mm Hg

With constant luminal diameter flow velocity is proportional to the driving pressure head, thus, a 15 % change in the pressure drop should result in a 15 % change in transit time With transit times about 10 s, the change would be about 1 5 s We may therefore, conclude that the observed initial change in intratubular pressure may cause a change in the measured proximal transit time of no more than 1 5 s which is within the error of a single measurement

The secondary elevation of 1—2 mm Hg in proximal pressure observed after a single dye injection occurred at a time when BP was stable no significant secondary pressure change occurred after saline injection, suggesting that it might be attributed to a specific effect of the dye A proximal intratubular pressure increment may be due to an increase in filtration pressure an inhibition of proximal reabsorptive capacity or to inhibition in reabsorptive capacity of Henle Loop and/or increase in the flow resistance of the loop The present data do not permit any definite distinction between these alternatives

The microperfusion data by Wilczewski *et al* (1970) suggest that LG at a concentration of 200 mg% on the luminal side completely blocked net fluid transport by the proximal tubule no effect could be demonstrated in perfused loops of Henle However Morel and Murayama (1970) found no serious interference with proximal reabsorption during perfusion with 100 mg% of LG The apparent difference between the results of Wilczewski *et al* and of others may of course be due to chemical differences in the Lissamine greens used even though neither the present in vivo study nor the in vitro study by Christensen and Frederiksen (1972) could demonstrate any significant difference in effects on isosmotic absorption with three chemically different LG dyes However several alternatives and more likely explanations are open to account for the difference in results Thus differences in pH were not excluded either since it was not reported whether or not the microperfusion fluids used by Wilczewski had been titrated to pH 7 4 after the addition of LG, finally technical artifacts likely to occur in microperfusion experiments cannot either be excluded as a possible cause of the disagreement in results However irrespective of the ultimate interpretation of the second pressure peak it will appear that the change in pressure as such (assuming flow resistance to be constant) was too small to influence significantly the measured loop transit time

Late and more prolonged effects occuring 15 minutes after a single LG injection were observed in the clearance experiments of group C The interpretation of these effects rests on the assumption that the functional parameters studied in superficial convolutions are quantitatively representative for the entire kidney Previous reports by *e g* Horster and Thurau (1968) Baines and de Rouffignac (1969) and Bonvalet (1970) have indicated functional heterogenity between superficial and juxtamedullary nephrons under the conditions of their experiments However using the modified Hansen technique described by Baines and de Rouffignac (1969) we were unable to detect any difference in the relative single nephron filtration rates between superficial and deep nephrons in the rats species used in our laboratory (Sprague Dawley) under the present control conditions nor were there any differences in

proximal tubular length between microdissected superficial and juxtamedullary nephrons in these rats (Elmer and Leyssac in preparation) Since the juxtamedullary nephron only amounts to 6 % of the total nephron population (de Rouffignac and Morel 1967) even minor functional differences would not invalidate the present conclusions

The most conspicuous functional change persistent throughout the second clearance period following a single injection of LG was an increase in osmotic excretion rate and urine flow rate a similar finding was obtained by Heller (1971) following repeated injections of LG It appears that the absolute rate of proximal reabsorption remained unchanged while inulin clearance and thus overall tubular reabsorption rate as well as glomerular filtration rate (GFR) increased about 24 % i e reabsorption rate increased in lower nephron segments distal to the proximal convolution The dead space of a rat kidney (BW 250 g) is about 15 μl (unpublished observation) with a urine flow of 4 μl/min or more it will be washed out by a volume at least about 4 times its own volume before the second clearance period starts (after about 15 min) Thus any dead space error would not seem to contribute significantly to the observed change in clearance Urine osmolality remained unchanged This fact excludes an anti vasopressinlike activity of LG as a contributing factor to the diuresis also wash-out of medullary hypertonicity due to a hypothetical increase in medullary blood flow can be ruled out as a cause by the same argument An osmotic effect of LG can also be ruled out as a cause of the diuresis At the beginning of the second clearance period the concentration of LG in urine was about 0 6—0 8 mM With 4 charged groups the osmotic activity would amount to no more than 2—3 mOsm At a pH below 7 it would be less A possible explanation would be that the loop of Henle would be responsible for the major increase in overall reabsorption rate

In conclusion the present results have failed to disclose any immediate change in tubular reabsorptive function following a single LG injection However 5—10 min later the functional state of the kidney was changed significantly as measured in clearance periods of 15—30 min This seems to indicate a delayed functional adjustment a phenomenon which is well known to occur also following other stimuli such as *e g* acute saline infusion (Bojesen 1954) This fact should limit the use of LG transit time measurements in physiological studies to a single injection (or a few repeated injections within a short time interval) at the end of the experiment

Thanks are due to Drs E Bojesen P Christensen and O Frederiksen [illegible] suggestion and criticism during preparation of the manuscript

The present investigation was supported by grants from Statens Lægevidenskabelige Forskningsråd and P Carl Petersens Fond

References

Baines A D and C de Rouffignac Functional heterogeneity of nephrons II Filtration rates intraluminal flow velocities and fractional water reabsorption *Pflügers Arch ges Physiol* 1969 *308* 260—276

Bojesen E A method for determination of inulin in plasma and urine *Acta med* [illegible] 1952 Suppl *266* 275—282

BOJESEN E The renal mechanism of dilution diuresis and salt excretion in dogs *Acta physiol scand* 1954 *32* 129—147

BOJESEN E and P P LEYSSAC Proximal tubular reabsorption in the rat kidney as studied by the occlusion time and Lissamine green transit time technique *Acta physiol scand* 1969 *76* 213—235

CHRISTENSEN P and O FREDERIKSEN The effect of some Lissamine greens on gall bladder fluid absorption and frog skin sodium transport *in vitro* *Pflugers Arch ges Physiol* 1972 *331* 160—171

DE ROUFFIGNAC C and J P BONVALET Étude chez le rat des variations du debit individuel de filtration glomerulaire des nephrons superficiels et profonds en fonction de l apport sode *Pflugers Arch ges Physiol* 1970 *317* 141—156

DE ROUFFIGNAC C and F MOREL Étude par microdissection de la distribution et de la longueur des tubules proximaux dans le rein de cinq especes de rongeurs *Arch Anat micr Morph exp* 1967 *56* 123—131

DORGE A and W NAGEL Die Wirkung von Lissamingrun auf den Natriumtransport an der isolierten Bauchhaut von Rana temporaria *Pflugers Arch ges Physiol* 1969 *313* 11—18

GERTZ K H J A MANGOS G BRAUN and H D PAGEL On the glomerular tubular balance in the rat kidney *Pflugers Arch ges Physiol* 1965 *285* 360—372

GOTTSCHALK C W and M MYLLE Micropuncture study of pressures in proximal tubules and peritubular capillaries of the rat kidney and their relation to ureteral and renal venous pressure *Amer J Physiol* 1956 *185* 430—439

HELLER J The influence of Lissamine green on tubular reabsorption of electrolytes and water in rats *Pflugers Arch ges Physiol* 1971 *323* 27—33

HORSTER M and K THURAU Micropuncture studies on the filtration rate of single superficial and juxtamedullary glomeruli in the rat kidney *Pflugers Arch ges Physiol* 1968 *301* 162—181

LEYSSAC P P Dependence of glomerular filtration rate on proximal tubular reabsorption of salt *Acta physiol scand* 1963 *58* 236—242

LEYSSAC P P The in vivo effect of angiotensin on the proximal tubular reabsorption of salt in rat kidneys *Acta physiol scand* 1964 *62* 436—448

LEYSSAC P P Some characteristics of the proximal tubular wall related to reabsorption during luminal occlusion following interruption of glomerular filtration *Acta physiol scand* 1965 *63* 36—45

MOREL F and Y MURAYAMA Simultaneous measurement of unidirectional and net sodium fluxes in microperfused rat proximal tubules *Pflugers Arch ges Physiol* 1970 *320* 1—24

RAMSAY J A and H J BROWN Simplified apparatus and procedure for freezing point determinations upon small volumes of fluid *J sci Instrum* 1955 *32* 372—376

LOCH RAMEL F and N JOTTERAND Natriuretic effect of Lissamine green *Experientia* (Basel) 1970 *26* 683

STEINHAUSEN M I IRAVANI G E SCHUBERT and R TAUGNER Auflichtmikroskopie und Histologie der Tubulusdimensionen bei verschiedenen Diuresezustanden *Virchows Arch path Anat* 1963 *336* 503—527

WILCZEWSKI T W H SONNENBERG and G CARRASQUER Permeability of superficial proximal tubules and loops of Henle to urea in rats *Proc Soc exp Biol* (N Y) 1970 *135* 609—619

Acta physiol scand 1973 87 307—319

From the Institute of Physiology University of Aarhus Denmark

The Effect of Noradrenaline and Adrenaline on Hepatosplanchnic Hemodynamics, Functional Capacity of the Liver and Hepatic Metabolism

By

NIELS KRARUP

Received 1 June 1972

Abstract

KRARUP N *The effect of noradrenaline and adrenaline on hepatosplanchnic hemodynamics functional capacity of the liver and hepatic metabolism* Acta physiol scand 1973 87 307—319

Cats in the postabsorptive state and anesthetized with chloralose were used for the experiments. Following a control period noradrenaline and adrenaline at doses of 0.5, 1.0, 2.0 and 5.0 μg/kg/min were infused continuously for about an hour into a femoral vein. Noradrenaline caused a pronounced constriction of the hepatic artery and a slight increase in the mesenteric vascular resistance. Small doses of adrenaline had a slight dilatory effect on the hepatic artery and mesenteric vessels whereas the largest dose caused a slight constriction in both these vascular beds. Neither of the drugs changed the portal venous resistance. The catecholamine infusions resulted in hepatic glycogenolysis and glycolysis but neither the splanchnic elimination of ethanol, the secretion and composition of bile, excretion of Indocyanine Green nor the splanchnic oxygen consumption were affected, indicating that the number of sinusoids perfused was not changed. A slight effect on the hepatic clearance and extraction ratio of Indocyanine Green was however observed. The results indicate that although the infusion of catecholamines in the present doses is accompanied by marked changes in hepato-splanchnic hemodynamics and liver metabolism, the intrahepatic distribution of blood flow is not subject to gross alterations. The existance of catecholamine dependent sphincter activity in the hepatic sinusoids observed in transillumination studies have not been confirmed by the present experiments.

Transillumination studies have revealed that catecholamines may change the intrahepatic distribution of blood flow causing a decrease in the number of perfused sinusoids (Wakim 1944, Seneviratne 1950, McCuskey 1966, Ho 1972). This concept has to some extent been supported by Daniel and Prichard (1951) in a radio angiographic study. Furthermore slight cooling changes liver function in a way which may be explained by a redistribution of liver blood flow elicited by means of an increased sympathetic tone (Larsen 1971, Krarup and Larsen 1972).

From the litterature it appears that little is known about a possible relationship between changes in hepatosplanchnic vascular parameters and liver function and only few experiments concerning the effect of catecholamines on liver function have appeared.

A decrease in the number of perfused sinusoids must be accompanied by a corresponding decrease in the functional capacity of the liver or liver mass. The present experiments were designed to compare the effect of adrenaline and noradrenaline on the hepato-splanchnic hemodynamics with the effect on liver function as measured by the elimination rate of ethanol, the uptake and excretion of Indocyanine Green (ICG) and the secretion of bile. Furthermore, liver metabolism was followed by determining the splanchnic consumption of oxygen and uptake/output of glucose, lactate and pyruvate.

Methods

24 cats, fasted overnight, were anesthetized with chloralose (50 mg/kg) and an initial dose of Nembutal (30 mg). The abdomen was opened through a midline incision and catheterisation of a hepatic vein was performed via the external jugular vein and the right heart. The catheter was manually guided into a liver lobe. In 14 of the experiments the cystic duct was ligated and the choledocus cannulated for collection of bile. In the remaining experiments the hepatic arterial and portal venous flows were measured simultaneously by means of electromagnetic flowmeters (Nycotron, Oslo). In these cats the common hepatic artery was dissected free and the right gastroduodenal artery ligated. After removal of connective and nervous tissue surrounding the artery, a flow probe (I.D. 1 mm) was placed around it. The portal vein was exposed, cleaned and supplied with a flow probe (I.D. 3 mm). Zero adjustment was performed according to the manufacturer's manual and calibration was made on the femoral artery and abdominal aorta.

In all experiments mean arterial blood pressure was recorded from a femoral artery, and in 6 of the experiments, in which portal and hepatic arterial flow was measured, also the portal and hepatic venous pressure was measured. Condensator manometers were used for pressure registration, and flows and pressures were recorded on a Berkman S II Dynograph. The femoral veins were used for infusions. Blood samples drawn from a femoral artery and a [hepati]c vein and bile samples were collected every 15 min after a recovery period of 90 min after the operation. About 150 min after the operation the catecholamine infusions were started. The body temperature was kept at 38.5 °C by gentle heating.

Estimation of the functional capacity of the liver. As parameters reflecting the functional capacity of the liver the elimination rate of ethanol and the bile flow and composition were used. Under conditions comparable with the present ones the elimination rate of ethanol within wide limits is independent of the ethanol concentration and the hepatic blood flow (Larsen 1963). In the perfused rat liver it has been found that the bile flow reflects the functional capacity of the liver, being independent of changes in total liver blood flow, but dependent on the number of perfused sinusoids (Brauer, Leong and Holloway 1954).

Administration of ethanol, Indocyanine Green, taurocholate, noradrenaline and adrenaline. Ethanol was infused continuously (35 μmol/kg/min) after a priming dose (6.5 mmol/kg) resulting in concentrations which saturate the elimination capacity. Indocyanine Green (ICG) (Hynson, Westcott and Dunning) was given as a priming dose (300 μg/kg) followed by a continuous infusion of 5 μg/kg/min which resulted in an almost horizontal time-concentration relationship. To compensate for the enterohepatic circulation of bile acids, taurocholate (Michrome) was given continuously at a rate of 0.20 μmol/kg/min. The priming doses of ethanol and ICG was dissolved in 0 ml isotonic NaCl. Ethanol, ICG and taurocholate used for continuous infusion were dissolved in isotonic NaCl 50 ml, to which albumin was added to increase the stability of the dye and the solution was protected against light. The infusion rate was 0.1 ml/min. Adrenaline and noradrenaline solutions were prepared just prior to infusion. The drugs were dissolved in isotonic NaCl, of which 0.1 ml/min was infused, the final infusion rates being 0.5, 1.0, 2.0 and 5.0 μg/kg/min.

Analytical procedures. Ethanol, plasma ICG and the biliary concentrations of ICG, bile acids and electrolytes were determined as previously described (Krarup and Larsen 1972). Hemoglobin was determined photometrically. The oxygen saturation was determined photometrically as described by Siggaard Andersen, Jorgensen and Naeraa (1962) except that the samples were hemolyzed prior to centrifugation. This was necessary because hemolyzing the centrifuged samples caused development of insoluble crystals probably of hemoglobin. The constants used for the calculation of the saturation per cent were determined in fully reduced and fully oxygenated cat blood. The constants determined did not differ significantly from

values for human blood Blood glucose was determined enzymatically by means of glucose oxidase (GLOX KABI) Blood lactate and pyruvate were determined according to Olsen (1971)

Calculations The total elimination rate of ethanol was calculated as the amount infused per minute corrected for the amount disappeared from or retained in the solvent space set as 65 % of the body weight (Larsen 1963)

The splanchnic elimination of ethanol consumption of oxygen and uptake/output of pyruvate lactate and glucose were calculated by multiplying the arterio-hepatic venous differences by the estimated hepatic blood flow (EHBF) The latter was determined by means of ICG using the Fick principle The oxygen concentrations in arterial and hepatic venous blood were calculated from the saturation per cent and the hemoglobin concentration assuming an oxygen binding capacity of 1 34 ml/g hemoglobin

Liver function was also followed by calculating the plasma clearance and extraction ratio of ICG Clearance was calculated as the amount of ICG infused corrected for the amount retained in the plasma volume divided by the mean arterial concentration and the extraction ratio as the arterio-hepatic venous ICG difference divided by the arterial concentration The biliary excretions of ICG and bile acids were calculated as the bile flow multiplied by the concentration in question

The vascular resistances were calculated in peripheral resistance units (PRU_4) after correction for the viscosity factor due to the hematocrite in the following manner

$$SVR = \frac{BP \times 60}{EHBF \times \eta_{rel}}$$

$$HAR = \frac{(BP - VP) \times 60}{AF \times \eta_{rel}}$$

$$MVR = \frac{(BP - PP) \times 60}{PF \times \eta_{rel}}$$

$$PVR = \frac{(PP - VP) \times 60}{PF \times \eta_{rel}}$$

(SVR splanchnic vascular resistance HAR hepatic arterial resistance MVR mesenteric vascular resistance PVR portal venous resistance BP mean arterial pressure mm Hg PP portal venous pressure mm Hg VP hepatic venous pressure mm Hg AF hepatic arterial flow ml/min PF portal venous flow ml/min η_{rel} relative viscosity calculated from the hematocrit using the results of Pirofsky (1953)

Statistical procedures The significancy of the effects of the catecholamines was tested by the method of paired comparisons using Student t test

Results

Hepatosplanchnic hemodynamics From Table I it appears that noradrenaline at infusion rates of 2 and 5 μg/kg/min decreases EHPF whereas smaller infusion rates are without effect Noradrenaline infused at rates higher than 0 5 μg/kg/min however caused an increase in hematocrit (from 0 37—0 42) so that EHBF was less effected The EHBF in the control periode was 41 ml/kg/min (S E = 2 0) Adrenaline at all infusion rates given caused no significant changes in EHPF

In 10 expts the hepatic and portal venous flow were determined electromagnetically 2 expts are illustrated in Fig 1 and 2 It will be noticed that infusion of catecholamines causes immediate changes in flow and pressure and steady levels are not reached until 15 min after the start of the infusion The results are listed in Table II It appears that directly determined liver blood flow (AF + PF) in steady state corresponds well with the indirect measurements of flow both before and after catecholamine infusion In general however the indirect measurements gave about 10 % higher values Noradrenaline in all doses given decreases arterial flow and

TABLE I The effect of different doses of noradrenaline and adrenaline on ethanol elimination hepatic uptake of ICG and hepatic plasma flow The numbers in brackets indicates the total elimination rate of ethanol as determined from the arterial elimination curve

		Splanchnic elimination of ethanol µmol/kg/min		Plasma clearance of ICG ml/kg/min		Extraction of ICG per cent		Estimated hepatic plasma flow ml/kg/min	
		A	B	A	B	A	B	A	B
Noradrenaline									
0 5 µg/kg/min	n = 3	30 (36)	32 (36)	5 5	4 7	20	18	28	31
1 0	n = 2	23 (33)	23 (31)	4 5	3 8	28	27	17	15
2 0	n = 4	30 (39)	29 (34)	6 4	4 6	19	22	27	21
5 0	n = 4	33 (40)	32 (40)	5 0	3 3	17	18	30	20
mean		30 (38)		[illegible] 5		20		27	
S E		1 8 (1 6)		[illegible] 4	—	1 7		2 3	
Adrenaline									
0 5 µg/kg/min	n 3	33 (40)	31 (40)	4 3	4 0	16	14	27	29
1 0	n = 2	37 (38)	38 (41)	4 3	4 2	15	15	29	30
2 0	n = 3	37 (40)	40 (42)	5 1	5 0	22	17	27	33
5 0	n = 3	29 (33)	29 (32)	4 5	3 3	20	17	20	21
mean		34 (38)		4 6		19		25	
S E		1 › (1 5)		0 3		1 0		2 3	

A mean value during the control period
B mean value during the drug infusion period
n number of experiments

except for one experiment with high infusion rate noradrenaline increases portal flow Adrenaline given at 5 µg/kg/min decreases flow both in the hepatic artery and portal vein but at smaller infusion rates the effect is slight and variable

The effect of catecholamines on mean arterial blood pressure was dependent on the drug and the dose given Noradrenaline in all doses increased BP the larger doses having the greatest effect Adrenaline infused at rates of 0 5 and 1 0 µg/kg/min caused an immediate decrease in BP which generally was normalized after 15 min (Fig 2) Higher doses of adrenaline caused an increase in BP which was somewhat smaller than when corresponding doses of noradrenaline was given The portal pressure always varied in parallel with portal flow (Fig 1 and 2) and the hepatic venous pressure was not significantly affected by the catecholamines

The splanchnic vascular resistance was increased by noradrenaline in all doses given This was due to a 200—400 % increase in hepatic arterial resistance and a slight (0—40 %) increase in mesenteric vascular resistance whereas the portal venous resistance remained unaltered Adrenaline at rates up to 2 0 µg/kg/min caused a slight decrease in splanchnic vascular resistance (15 ± 6 % mean and S E), and at a rate of 5 0 µg/kg/min a 25 % increase in splanchnic vascular resistance was found The smaller doses of adrenaline decreased the hepatic arterial resistance (0—20 %) whereas doses higher than 1 0 µg/kg/min caused a slight increase (15—40 %) The mesenteric vascular resistance decreased (0—50 %) when

Fig 1 The effect of continuous infusion of noradrenaline (5 μg/kg/min) on mean arterial and portal venous pressure and mean hepatic arterial and portal venous flows The infusion is started at the arrow

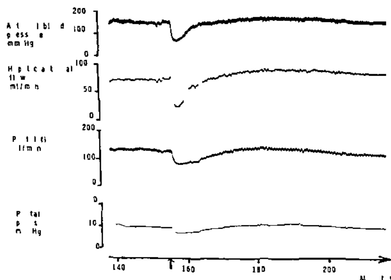


Fig 2 The effect of continuous infusion of adrenaline (1 μg/kg/min) on mean arterial and portal venous pressure and mean hepatic arterial and portal venous flows. The infusion is started at the arrow

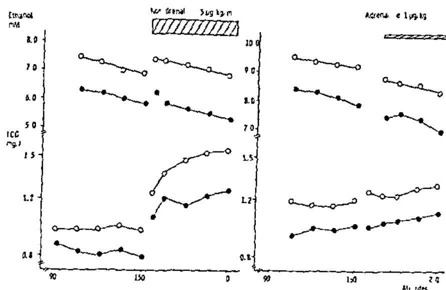


Fig 3 The effect of continuous infusions of noradrenaline (5 μg/kg/min) and adrenaline (1 μg/kg/min) on the arterial (O) and hepatic venous (●) blood concentrations of ethanol and plasma concentrations of ICG

TABLE II The effect of different doses of noradrenaline and adrenaline on portal venous hepatic arterial and total (portal+hepatic arterial) liver blood flow and estimated hepatic blood flow

	Portal venous blood flow ml/kg/min		Hepatic arterial blood flow ml/kg/min		Total liver blood flow ml/kg/min		Estimated hepatic blood flow ml/kg/min	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Noradrenaline								
0.5 μg/kg/min	24	28	10	6	34	34	35	34
2.0	27	31	19	11	46	42	50	44
5.0	41	50	19	10	60	60	64	54
5.0	23	20	9	4	32	24	35	22
Adrenaline								
0.5 μg/kg/min	29	30	11	12	40	42	48	53
0.5	21	19	10	9	31	28	45	56
1.0	38	37	19	24	57	61	56	60
1.0	26	36	10	10	36	46	40	47
2.0	18	29	7	6	25	35	31	39
5.0	29	27	23	18	52	45	50	47
mean	28		14		41		45	
S.E.	2.3		1.8		3.8		3.3	

A Mean value during the control period
B Steady state value during the drug infusion period

TABLE III The effect of different doses of noradrenaline and adrenaline on bile flow and excretion of ICG and bile acids

	Bile flow µl/kg/min		ICG recovery per cent		Excretion of bile acids µmol/kg/min	
	A	B	A	B	A	B
Noradrenaline n = 8	15.8 ± 1.4	15.3 ± 1.6	98 ± 3	95 ± 2	0.36 ± 0.02	0.35 ± 0.01
	$0.30 < p < 0.40$		$0.05 < p < 0.10$		$0.60 < p < 0.70$	
Adrenaline n = 6	11.3 ± 2.0	9.7 ± 1.9	96 ± 3	93 ± 4	0.32 ± 0.04	0.31 ± 0.04
	$0.05 < p < 0.10$		$0.20 < p < 0.30$		$0.05 < p < 0.10$	

A mean and S.E. during the control period
B mean and S.E. during the drug infusion period
n number of experiments

TABLE IV The effect of different doses of noradrenaline and adrenaline on splanchnic oxygen consumption arterial blood glucose and splanchnic output of glucose

	Splanchnic oxygen consumption µmol/kg/min		Arterial glucose concentration mg			Splanchnic glucose output mg/kg/min		
	A	B	A	C	D	A	C	D
Noradrenaline								
1.0 µg/kg/min n = 2	110	113	120	128	123	1.4	3.4	0.7
5.0 n = 2	137	134	184	156	247	3.0	6.4	20.9
Adrenaline								
0.5 µg/kg/min n = 3	138	134	154	148	221	2.2	5.7	5.4
1.0 n = 2	125	129	149	270	293	1.6	21.2	7.0
2.0 n = 3	120	127	161	231	345	2.0	20.6	9.9
5.0 n = 3	113	105	105	186	193	1.5	17.1	2.8

A Mean value during the control period
B Mean value during the drug infusion period
C Mean value 15 min after the drug infusion was started
D Mean value 60 min after the drug infusion was started
n number of experiments

adrenaline was infused except when given at the highest rate Neither adrenaline changed the portal venous resistance significantly

Liver function elimination of ethanol ICG and secretion of bile The effect of a large pressor dose of noradrenaline and a depressor dose of adrenaline on the elimination rate of ethanol and the hepatic uptake of ICG is shown in Fig 3 It appears that in neither of the experiments is the slope of the elimination curve of ethanol altered but there is slight changes in the arterial concentration levels The increase in the arterio-hepatic venous ethanol difference in the noradrenaline experiment corresponds to the decrease in liver blood flow as reflected in the increase in ICG extraction

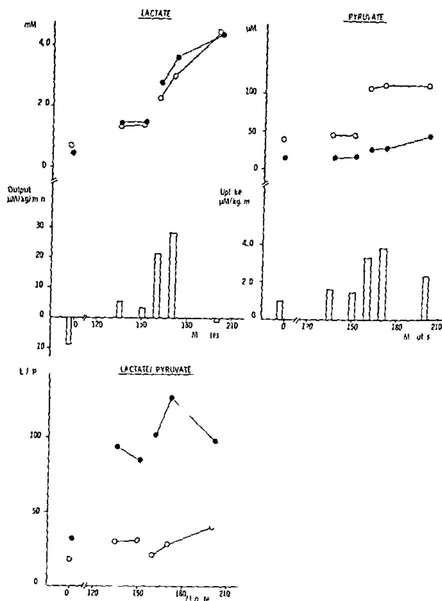


Fig 4 The effect of adrenaline (5 ug/kg min) on the arterial (O) and hepatic venous (●) blood concentrations of lactate and pyruvate the splanchnic output or uptake of lact te and pyruvate and the lactate/pyruvate ratios in arterial (O) and hepatic enous (●) blood

From Table I it appears that in the doses used neither of the drugs causes significant changes in the elimination rate of ethanol There is a significant decrease in the plasma clearance of ICG in the noradrenaline as well as adrenaline experiments noradrenaline having the greatest effect The extraction ratio of the dye is not changed by noradrenaline whereas adrenaline causes a decrease in the extraction ratio Except for a temporary (about 15 min) slight (10—15 %) depres

sive effect noradrenaline even at the highest doses did not change the bile flow or the excretion rates of ICG and bile acids (Table III) whereas adrenaline caused a slight decrease in bile flow without changing the ICG and bile acid excretion It will be noticed that the bile acid excretion exceeds the amount of bile acids infused per min (0 20 μmol/kg/min) The electrolyte composition of the bile was deter mined in 8 of the experiments and the following results were obtained in the control period Na 161 meq/l, K 4 1 meq/l Cl 102 meq/l and HCO_3 23 meq/l Neither of the drugs caused significant effects on the electrolyte composition of the bile

Hepatic metabolism From Table IV it appears that neither of the drugs alter the splanchnic oxygen consumption Except when the smallest dose of noradrenaline is given there is an increase in the blood glucose corresponding to the increase in the splanchnic output of glucose Compared with infusion of noradrenaline the effect is larger and starts earlier when adrenaline is infused

Fig 4 shows the lactate and pyruvate concentrations from an experiment in which adrenaline 5 0 μg/kg/min was infused after the control period The priming dose of ethanol increases the lactate concentrations in arterial and hepatic venous blood and the splanchnic uptake of lactate is reversed to an output The pyruvate con centrations are almost unaltered hence the lactate/pyruvate ratio is changed to a more reduced state by ethanol The adrenaline infusion causes a pronounced in crease in the splanchnic output of lactate and the lactate concentrations The pyruvate concentrations also increases after the adrenaline infusion although the splanchnic area takes up more pyruvate than in the control period The lactate/pyruvate ratios in arterial and hepatic venous blood are not significantly changed Lactate and pyruvate were determined in three more experiments in which adrenaline 1 0 μg/kg/min and noradrenaline 1 0 and 5 0 μg/kg/min were infused and qualita tively similar results were obtained

Discussion

Hepatosplanchnic hemodynamics The flow values found in the present experiments are in perfect agreement with the values generally given for cats (Greenway and Stark 1971) The difference between the EHBF and the directly measured flow may be due to methodological errors especially the calibration of the probes and a non representative hepatic venous sampling Also flow through accessory hepatic arteries which are often found (Michels 1955) may account for the discrepancy

In studies of the effect of catecholamines on EHBF variable results have been obtained Generally as in these experiments noradrenaline increased and small doses of adrenaline decreased the splanchnic vascular resistance (Bearn Billing and Sherlock 1951 Smythe Gilmore and Hanford 1954 Farrand Larsen and Horvath 1957) The decrease in hepatic arterial flow despite the increase in BP after nor adrenaline also agrees with previous findings (Greenway Lawson and Mellander 1967 Ross and Kurrasch 1969) and indicates constriction of hepatic arterioles

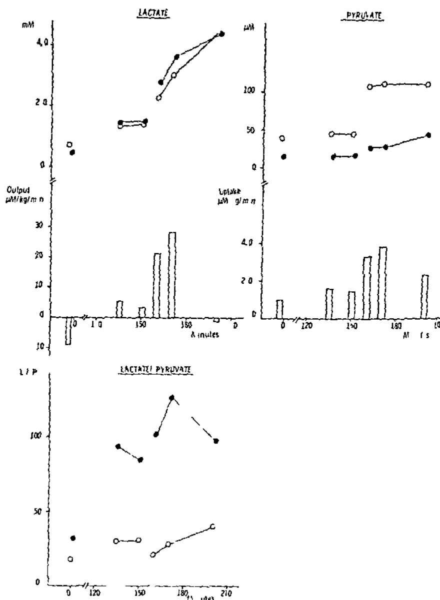


Fig 4 The effect of adrenaline (5 μg/kg min) on the arterial (O) and hepatic venous (●) blood concentrations of lactate and pyruvate the splanchnic output or uptake of lactate and pyruvate and the lactate/pyruvate ratios in arterial (O) and hepatic venous (●) blood

From Table I it appears that in the doses used neither of the drugs causes significant changes in the elimination rate of ethanol There is a significant decrease in the plasma clearance of ICG in the noradrenaline as well as adrenaline experiments noradrenaline having the greatest effect The extraction ratio of the dye is not changed by noradrenaline whereas adrenaline causes a decrease in the extraction ratio Except for a temporary (about 15 min) slight (10—15 %) depres

by a decrease in plasma clearance and an increase in hepatic extraction ratio of dyes decreases in plasmaflow having the reverse effects In the present experiments changes in plasma clearance and extraction ratio of ICG was observed which can not be merely due to the changes in hepatic plasmaflow induced by the catechol amines This may indicate an effect of catecholamines on the dye eliminating func tion of the liver A similar but larger effect of catecholamines on the extraction of colloid gold in the perfused rat liver was demonstrated by Brauer (1958) who proposed that the findings might be due to a decrease in the number of perfused sinusoids caused by catecholamine dependent sinusoidal sphincters This possibility is not likely in the present experiments as no change in the ethanol elimination and hepatic excretory function was observed

3 *Bile flow and composition* Studies in dogs (Tanturi and Ivy 1937) and rabbits (Archdeacon Danforth and Dummit 1954 Kjellgren 1954) have shown a depres sive effect of pressor doses of catecholamines on the bile flow smaller doses being without significant effect It has further been stated that the ductular fraction of bile is dependent of the hepatic arterial flow (Wheeler and Mancusi Ungaro 1966) In the present experiments infusion of even the highest dose of noradrenaline which decreased the hepatic arterial flow caused but a slight and transient decrease in bile flow and the composition of the bile was not affected by the catecholamines indicating that the hepatic excretory function remained unaltered

Hepatic metabolism In man the splanchnic oxygen consumption was increased by adrenaline (Bearn *et al* 1951) and in dogs by noradrenaline (Smythe *et al* 1954) It was suggested that the effect of adrenaline was due to hepatic elimination of lac tate liberated in the periphery (Bearn *et al* 1951) This may explain the unaltered splanchnic oxygen consumption after catecholamines in the present experiments as the redox shift induced by ethanol resulted in inability of the liver to clear lactate However also Farrand *et al* (1957) were unable to demonstrate an effect of nor adrenaline on splanchnic oxygen consumption The rise in arterial glucose lactate and pyruvate concentrations and splanchnic output of glucose and lactate agrees with the glycogenolytic and glycolytic actions of the hormones The lack of effect of the catecholamines on hepatic venous lactate/pyruvate ratio further support the concept of an even distribution of blood flow on the hepatic sinusoids as an in adequate perfusion would be reflected in an increase in the cytoplasmic redox level (Oliva 1969)

Conclusion

The experiments have shown that infusion of catecholamines at rates which elicit well known metabolic responses in the liver is accompanied by changes in flow and resistances in the hepatic artery and mesenteric vessels whereas no effect on the portal venous resistance was found The hemodynamic and metabolic effects were not coupled to significant changes in the functional capacity of the liver and no signs of inadequate hepatic perfusion were found

In contrast to transillumination studies and radioangiographic observations the present experiments indicate that the distribution of blood flow on the hepatic sinusoids is not subject to gross alterations when catecholamines are infused Furthermore, the experiments seem to rule out the possibility that catecholamine are responsible for the effect of slight hypothermia on liver function

The technical assistance of Miss I Abildgaard Mrs L Nielsen and Mrs J Justesen is gratefully acknowledged

References

ANDREWS W H H R HECKER and B G MAEGRAITH The action of adrenaline acetylcholine and histamine on the perfused liver of the monkey cat and rabbit *J Physiol* (London) 156 *132* 509—521

ARBAB-ZADEH A Die Rolle der Nebennieren bei Alkoholtoleranz aus forensischer Sicht *Med Welt* 1969 *20* 461—464

ARCHDEACON J W J T DANFORTH and G D DUMMIT Factors affecting bile flow in the rabbit and rat *Amer J Physiol* 1954 *178* 499—502

BEARN A G B BILLING and S SHERLOCK The effect of adrenaline and noradrenaline on hepatic blood flow and splanchnic carbohydrate metabolism in man *J Physiol* (London) 1951 *115* 430—441

BRAUER R W Blood flow distribution in the liver and the circulatory control of liver function In *Liver function* Waverly Press Baltimore 1958 113—127

BRAUER R W C F LEONG and R J HOLLOWAY Mechanics of bile secretion *Amer J Physiol* 1954 *177* 103—113

CLARK W C and P A OWENS The influence of glucocorticoid epinephrine and glucagon on ethanol metabolism in the dog *Arch int Pharmacodyn* 1966 *16_* 355—363

DANIEL P M and M M L PRICHARD Effects of stimulation of the hepatic nerves and of adrenaline upon the circulation of the portal venous blood within the liver *J Physiol* (London) 1951 *114* 538—548

DEAL C P and H D GREEN Comparison of changes in mesenteric resistance following splanchnic nerve stimulation with responses to epinephrine and norepinephrine *Circulat Res* 1956 *4* 38—44

DRESEL P and J WALLENTIN Effects of sympathetic vasoconstrictor fibres noradrenaline and vasopressin on the intestinal vascular resistance during constant blood flow or blood pressure *Acta physiol scand* 1966 *66* 427—436

FARRAND E A R LARSEN and S M HORVATH Effects of L epinephrine and L norepinephrine on the splanchnic bed of intact dogs *Amer J Physiol* 1957 *189* 576—579

GREENWAY C V A E LAWSON and S E MELLANDER The effect of stimulation of the hepatic nerves infusions of noradrenaline and occlusion of the carotid arteries on liver blood flow in the anesthetized cat *J Physiol* (London) 1967 *18?* 21—41

GREENWAY C V and A F LAWSON *β* adrenergic receptors in the hepatic arterial bed of the anesthetized cat *Canad J Physiol* 1969 *47* 415—419

GREENWAY C V and R D STARK Hepatic vascular bed *Physiol Rev* 1971 *51* ?3—65

HO M S L Responses of hepatic microvessels *Quart J exp Physiol* 1972 *57* 226—232

KJELLGREN K Biliary secretion in relation to the liver temperature and the blood pressure *Acta Soc Med upsalien* 1954 *59* 172—185

KLINGMAN G J R BANE and H B HAAG Studies on severe alcohol intoxication in dogs III Effect of adrenalectomy *Quart J Stud Alcohol* 1959 *20* 13—23

KRARUP N and J A LARSEN The effect of slight hypothermia on liver function as measured by the elimination rate of ethanol the hepatic uptake and excretion of Indocyanine Green and bile formation *Acta physiol scand* 1972 *84* 396—407

LARSEN J A Elimination of ethanol as a measure of the hepatic blood flow in the cat. *Acta physiol scand* 1963 *57* 209—223

LARSEN J A The effect of cooling on liver function in cats *Acta physiol scand* 1971 *81* 197—207

MCCUSKEY, R. S A Dynamic and static study of hepatic arterioles and hepatic sphincters. *Amer J Anat* 1966 *119* 455—478

MICHELS N A. *Blood supply and anatomy of the upper abdominal organs with a descriptive atlas* Lippincott, Philadelphia, Pennsylvania 1955

OLSEN C An enzymatic fluorometric micromethod for the determination of acetoacetate β hydroxybutyrate pyruvate and lactate *Clin chim Acta* 1971 *33* 293—300
OLIVA P B Lactic acidosis. *Amer J Med* 1970 *48* 209—215
PIROFSKY B The determination of blood viscosity in man by a method based on Poiseuille s law *J clin Invest* 1953 *32* 292—298
ROSS G Escape of mesenteric vessels from adrenergic and noradrenergic vasoconstriction. *Amer J Physiol* 1971 *221* 1217—1222
ROSS G and M KURRASCH Adrenergic re ponses of the hepatic circulation *Amer J Physiol* 1969 *216* 1380—1385
SCHOLTHOLT J W LOCHNER H RENN and T SHIRAISHI Die Wirkung von Noradrenaline Adrenaline Isoproterenol und Adeno in auf die Durchblutung der Leber und des Splanchnicus Gebiete des Hundes *Pflugers Arch ges Physiol* 1967 *295* 129—154
SENEVIRATNE R D Physiological and pathological responses in the blood vessels of the liver *Quart J exp Physiol* 1950 *35* 77—110
SIGGAARD-ANDERSEN O K JORGENSEN and N NERAA Spectrophotometric determination of oxygen saturation in capillary blood *Scand J clin Lab Invest* 1962 *14* 298—302
SMYTHE C McC J P GILMORE and S W HANDFORD The effect of levarterenol (L-nor epinephrine) on hepatic blood flow in the normal anesthetized dog *J Pharmacol exp Ther* 1954 *110* 398—402
STOKES P E and B LASLEY Further studies on blood alcohol kinetics in man as affected by thyroid hormones insulin and D glucose In *Biochemical factors in alcoholism* Pergamon Press Oxford 1967
TANTURI C A and A C IVY A study of the effect of vascular changes in the liver and the excitation of its nerve supply on the formation of bile *Amer J Physiol* 1937 *121* 61—74
WAKIM K G The effect of certain substances on the intrahepatic circulation of blood in the intact animal *Amer Heart J* 1944 *27* 289—300
WINKLER K and C GRAM The kinetic of bromsulphalein elimination during cotninuous in fusion in man *Acta med scand* 1965 *178* 439—452
WHEELER H O and P L MANCUSI UNGARO Role of bile ducts during secretin choleresis in dogs *Amer J Physiol* 1966 *210* 1153—1159

Acta physiol scand 1973 87 320—333

From the Institute of Information Transmission Problems The Academy of Science of the USSR Moscow

On the Descending Control of the Lumbosacral Spinal Cord from the "Mesencephalic Locomotor Region"

By

S GRILLNER[1] and M L SHIK

Received 5 June 1972

Abstract

GRILLNER S and M L SHIK *On the descending control of the lumbosacral spinal cord from the mesencephalic locomotor region* Acta physiol scand 1973 87 320—333

Continuous stimulation (60 c/s) of a region below the inferior colliculus can induce locomotion on the treadmill of precollicular postmammilar cats This study aims at revealing what changes occur in the spinal cord when the locomotor region is stimulated This stimulation enables the cat to walk if the treadmill is moved After controlling the threshold for evoking good locomotion the cats were curarized Stimulation at a strength that evoked walking prior to curarization induced a depression of inhibitory short latency reflex effects to α motoneurones from cutaneous and high threshold muscular afferents without changing the direct excitability of α motoneurones The threshold for evoking longlasting reciprocally organized discharges was lowered The results suggest that the effects are induced by a slow fiber system that releases the activity of the spinal stepping generating neurones The results would be explained if the noradrenergic reticulospinal system was activated from the mesencephalic locomotor region

Data from different classes of vertebrates show that the nervous machinery for generating alternate activation of different muscle groups in locomotion is located in the spinal cord (Gray 1950 von Holst 1935 T G Brown 1911 1914 *cf* Bernstein 1967) The alternate activation of different muscles is essentially due to a central program (T G Brown 1911 1914 Lundberg 1969 b and Szekely *et al* 1969), although a nonspecific cyclic afferent activity might play a large role as well (Gray 1950 Shik *et al* 1966 b)

The chronic spinal cat performs spinal stepping while the acute spinal cat will walk only under particular circumstances such as during nociceptive stimulation of the perineal region (Sherrington 1910) during high frequency electrical stimulation of a certain point on the transverse section of the cervical spinal cord (Roaf and Sherrington 1910) or after an i v injection of DOPA (Grillner 1969 b Budakova 1971) The EMG pattern of spinal stepping is not sufficiently known at present and

[1] Present address Dept of Physiology Fack 400 33 Goteborg Sweden.

it cannot be excluded that stepping in the spinal cat is somewhat different from stepping in mesencephalic (Gambarian *et al* 1971) and intact cats (Engberg and Lundberg 1969)

The acute precollicular postmammilar cat cannot walk either spontaneously or under natural exteroceptive stimulation but acute premammilar (mesencephalic) or chronic postmammilar cats can (Hinsey Ranson and McNattin 1930 T G Brown 1941 P Bard M B Macht 1958 Woods 1964) In a mesencephalic cat locomotion can be induced if the region below the inferior colliculus is stimulated repetitively and the ground beneath the cat is set in motion with a treadmill Depending on the stimulation strength the cat changes from a diagonal pattern to a gallop At normal stimulation strength no locomotion is induced by the stimulation in itself but only in combination with the movement of the treadmill inducing a cyclic afferent input from the limbs (Shik *et al* 1966 a b) Only at very high strength can rhythmic activity be evoked by the stimulation alone Since a change is induced which enables the cat to walk it seems reasonable to assume that the stimulation of the mesencephalic locomotor region releases the spinal circuits for alternate activation of flexors extensors in locomotion

With this background it seems meaningful to investigate what changes occur in the spinal cord when the midbrain locomotor region is stimulated to enable the cat to execute locomotion Mesencephalic cats were prepared and the threshold for evoking locomotion by stimulation was checked carefully, after this the cats were curarized which allowed a study of changes in the spinal cord without having either rhythmic activity or effects of proprioceptor activation (muscle spindles or Golgi tendon organs) due to various experimental procedures The investigation revealed that the short latency inhibitory transmission from afferents evoking the flexor reflex was depressed during stimulation of the locomotor region whereas activity in neuronal circuits similar to those revealed after DOPA (Jankowska *et al* 1967 a b) could be evoked more readily The results would be explained if one of the main effects of locomotor region stimulation is to activate the descending noradrenergic pathways enabling the spinal cord to respond with locomotion. This interpretation is in keeping with the ideas of an unindividualized mode of control developed theoretically by Tsetlin (1969) and Gelfand and Tsetlin (1971) Applied to locomotor control it would be predicted that a relatively simple descending control signal would change the matrix of the spinal cord to a circuitry capable of generating locomotion The first examples of descending systems causing such profound change in the spinal cord are the systems activated with DOPA or 5HTP iv (Lundberg 1966)

Methods

21 cats were operated partially under ether and subsequently decerebrated precollicula... postmammillarly (Shik *et al* 1966 see Fig 1 section II) A laminectomy was performed ... tween L4—L7 and the ventral root of L7 was cut and prepared for recording ... discharges Some of the nerves to gastrocnemius soleus (G S) or quadriceps (Q) or ... to flexor digitorum longus (FDL) (without the interosseus nerve) were preparated ... tion as well as the sural the contralateral hamstring nerves ...

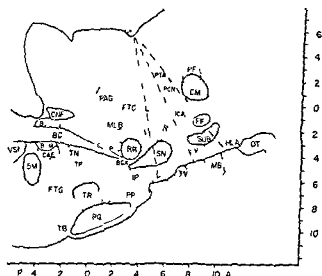


Fig 1 Schematic drawing of the midbrain from Bermans (1968) atlas of the cats brainstem The effective region for inducing locomotion is in and around the cuneiforme nucleus (CNF) The structures surrounded by interrupted lines are medial to a sagittal plane 4 mm lateral to the midline Structures with full line are in a plane of 4 mm lateral to the midline The sections indicated by straight dorsoventral interrupted lines refer to preparations with sections at these levels The most rostral preparation (premammillary) can exhibit spontaneous locomotion or at least locomotion on the treadmill whereas with the middle section (precollicular postmammillar) locomotion can be induced only by stimulation of the region surrounding nucleus cuneiforme (Shik *et al* 1966 1967) With the most caudal section no locomotion can be induced

The abbreviations are as follows BC brachium conjunctivum BCM Marginal nucleus of the br conjunctivum BCX decussatio brachium conjunctivum CAE nucleus caeruleus CM centrum medianum CNF nucleus cuneiformis 5M trigeminal motor nucleus FF nucleus area foreli FTC central tegmental field FTG Gigantocellular tegmental field HLA lateral hypothalamic area ICA interstitial nucleus of Cajal IP nucleus interpenduncularis MB mammillary bodies MLB medial longitudinal bundle OT tractus opticus PAG periaqueductal gray PC nucleus of the posterior commissure PF parafascicular nucleus PG pontine gray PP pes pedunculi PTA anterior pretectal nucleus R nucleus ruber RR retrorubral nucleus SN subst nigra SUB subthalamic nucleus TB trapezoid body 3N nervus oculomotorius TV ventral and dorsal tegmental nuclei TR tegmental reticular nucleus VSN nucleus vestibularis superior VTA ventral tegmental area of Tsai

semitendinosus The cat was mounted on a treadmill with the head fixed in a stereotactic device and the lumbar (L?—L3) vertebrae and sacrum were fixed A glass coated tungsten electrode with a tip of 0 μm was inserted in a Horsley Clarke plane (P2 L4) at a depth of between 5 5 6 5 mm below the surface of the inferior colliculus The electrode was left in a position in which locomotion could be induced at low threshold on the treadmill with the different gaits (10—90 μA monopolar stimulation at a frequency of 30 or 60 Hz and a pulse duration of 0 5—1 0 ms) It was also checked that the quality of locomotion was good *i e* by visual inspection looking at the interlimb coordination as well as the movement of the individual limb and its joints The eye is very sensitive in detecting even small abnormalities in gait The threshold for evoking locomotion was defined as the lowest stimulation strength at which a coordinated walk on the treadmill could be evoked Afterwards the cat was curarized (Flaxdil®) and respirated artificially The hindlimb nerves and the L7 ventral root were mounted for stimulation and recording respectively sometimes additional ventral roots were cut

The nerves were stimulated with pulses of 100 μs duration and recordings were monitored on a dual beam oscilloscope which could be photographed The body temperature was kept within 36 5—38 C as well as the two pools around the laminectomy and the hindlimb nerves

Continuous brainstem stimulation was no longer than 1 min and always followed by a 2 min period of rest Each test conditioning trial (peripheral nerves) was not repeated more often than once a second

Abbreviations G S gastrocnemius soleus Q quadriceps FDL the nerve to flexor digitorum longus and flexor hallucis longus with the nerve to interosseus removed while sometimes the nerves to m popliteus and tib posterior were included Sur sural nerve LO locomotor region

Results

a) *The effect of locomotor region stimulation on the size of the monosynaptic reflexes to hindlimb motoneurones*

In the curarized mesencephalic cat the size (amplitude) of the monosynaptic reflex evoked from the nerve to gastrocnemius soleus is virtually unchanged when the locomotor region is stimulated repetetively at a strength that resulted in good walking before the cat was curarized (cf Methods) This finding was confirmed in all experiments but if the strength was increased above this level (approximately 5—10 μA above the threshold value for locomotion) the monosynaptic reflex itself could change in a somewhat unpredictable way The size of the monosynaptic reflex would either be facilitated or inhibited These effects often outlasted the stimulation by several seconds or even minutes Similar effects were obtained with the knee flexor posterior biceps semitendinosus

Hence it is concluded that the direct (monosynaptic) excitability of hindlimb extensor and flexor motoneurones does not change significantly when the locomotor region is stimulated at a strength high enough to give walking before the curarization at higher strength of stimulation however the excitability does change

b) *The effect of locomotor region stimulation on the reflex transmission from cutaneous and muscular afferents to ipsilateral hindlimb α motoneurones*

The reflex effects evoked from peripheral nerves can be tested by measuring the size of a monosynaptic test reflex when it is preceded by a conditioning nerve volley (e g the sural nerve) at different intervals as in Fig 2 A (crosses) A conditioning volley to the sural nerve including only low threshold cutaneous afferents inhibits the monosynaptic test reflex from gastrocnemius soleus (G S) starting after 2 ms This reflex effect is evoked with such a short latency that it must represent a true spinal reflex with no involvement of spino bulbo pinal reflexes. When the same procedures are used during continuous stimulation in the mesencephalic locomotor region the inhibitory effect is entirely abolished Thus the short latency reflex transmission from the sural nerve is virtually blocked under locomotor region stimulation

In all preparations in which good locomotion was evoked from the brain stem the inhibitory effects evoked from the sural nerve could be reduced substantially by midbrain stimulation which is shown for 12 expts in Fig 2 B in which each vertical line indicates one test conditioning procedure in one experiment The crosses indicate the inhibitory effect prior to brainstem stimulation and the open circles during stimulation The graph also includes some experiments in which the reflex effects to a quadriceps (Q) and a flexor digitorum longus (FDL) test were examined It should be noted that a test volley to either Q or G S will activate mostly slow motor units since they get larger Ia EPSPs which is why predominantly inhibitory effects has been encountered although low threshold cutaneous afferents in the sural nerve give predominantly excitatory effects in fast G motoneurones (Burke J

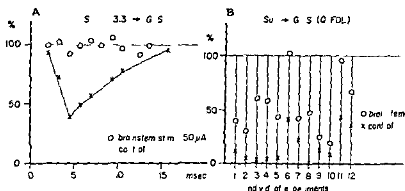


Fig 2 *Depression of transmission in short latency reflex arcs from the sural nerve to hindlimb extensor motoneurones* The graph to the left shows a gastrocnemius test reflex when conditioned by a volley in the sural nerve at different intervals before and during stimulation of the midbrain "locomotor region at 50 µA The abscissa shows the time interval between the incoming volley of the conditioning and the effective test volley The incoming volley was recorded at the dorsal root entry zone of L7 The right graph shows the size of a monosynaptic extensor test reflex when conditioned by a volley in the sural nerve in 12 different expts before and during locomotor region stimulation In exp 7 and 9 the test was evoked from FDL instead of G S and in exp 11 from Q

kowska and ten Bruggencate 1970) Also the inhibitory effects evoked from high threshold cutaneous afferents were regularly inhibited

To examine the reflex effects from muscle afferents the nerve to flexor digitorum longus (FDL) was used for conditioning Reflex effects evoked with less than 2 times threshold were never shown to be influenced while effects evoked with 4—7 times threshold were regularly depressed (Fig 3) These reflex effects were evoked at a latency short enough to exclude spino bulbospinal reflexes Note that the latency of these latter pathways when excited from the hindlimb is nearly 20 ms (Shimamura and Livingston 1963) These reflexes were consistently depressed by brainstem stimulation but to a lesser degree than those from the sural nerve It is evident that the afferent volley in FDL stimulated at this strength includes afferents from the secondary endings of the muscle spindle (Eccles and Lundberg 1959) but the possible contribution from other afferents with a similar diameter is at present unknown The inhibitory effects evoked in the control situation became larger if the stimulation strength was increased to include also group III afferents from muscle These additional effects could be similarly depressed during brain stem stimulation

In 2 expts when the knee flexor PBSt was used for testing the reflex effects evoked from the sural nerve and the nerves to G S and FDL all gave inhibition with a central delay of only 2—3 ms Such inhibitory effects can be evoked in decerebrate cats with a pontine lesion (Holmqvist and Lundberg 1961) The latter pathways cause an opening of the alternate spinal inhibitory pathways from the flexor reflex afferents and a blockade of the excitatory paths The observed short latency effects were similarly depressed by locomotor region stimulation as the corresponding inhibitory effects to extensors

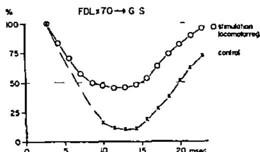


Fig 3 *Depression of transmission in short latency reflex arcs from FDL to G S* Representation identical with Fig 2 A A single conditioning volley to the nerve to FDL (interosseus nerve removed) was used at a stimulation strength including only group I and II afferents Effects from group I afferents were not affected

In conclusion synaptic effects from ipsilateral cutaneous and gr II and III muscular afferents were regularly depressed by stimulation of the locomotor region whereas no effects could be revealed on gr I volleys The locomotor region stimulation is effective with the same low stimulation strength as can be used to induce locomotion In cats in which no or very poor locomotion resulted from the stimulation no or very weak effects on the reflex transmission were evoked from the mesencephalic locomotor region and thus the two effects *i e* depression of reflex transmission and locomotion semeed to occur in parallel A normal locomotion with perfect movements occurs usually from a very small region (Shik *et al* 1966 a 1967) but from a more ventral region various autonomic effects (respiration circulation bladder) (Kuro 1965 Sirota *et al* 1971) are evoked as well as increasing muscular tone Usually no locomotor effects can be evoked from this region but under some conditions alternate movements of the limb occur which look like a kind of disturbed locomotion at a strength just suprathreshold for the locomotor effects from the appropriate region These effects can occasionally be evoked from a rather large area It cannot be excluded that the effective region for locomotion is larger than that described by Shik *et al* (1967) but that stimulation of the ventral region in addition activate several other systems resulting in a blockage or in a disturbance of the locomotion If the stimulation electrode was moved in a vertical track it was regularly found that 1 mm above the effective region no effects could be evoked on the reflex transmission with the same strength of stimulation At 1 or 2 mm below the most effective stimulation point effects on the reflex transmission were still evoked which however were smaller

c) *Longlasting discharges evoked with a central latency of 100—200 msec*

In the intercollicularly decerebrated cat a short train of impulses to a peripheral nerve at high strength can but only very rarely evoke a longlasting discharge in flexor filaments with a latency of 100—200 ms which builds up to a maximum after half a second or so (Engberg Lundberg and Ryall 1969) In this precollicular preparation we could regularly evoke similar discharges (from Sur G S FDL PBSt) in the 8 cats which were tested Two of these precollicular preparations were afterwards sectioned intercollicularly after which these late discharges could no

FDL 52



Brainstem 25µA 30pps

rest g



FDL 52



Fig 4 *Longlasting discharges evoked with a long central delay* In a filament to semitendinosus a short train of pulses to the nerve to FDL at high strength (52×thr) evoked no late effects whereas the same stimulation during stimulation of the locomotor region at 25 µA and 30 Hz evoked a discharge with a long latency (note time calibration) and of a very long duration This effect was evoked in 8 successive trials after stimulation whereas no such effects were evoked prior to the stimulation No changes in the direct excitability as judged by monosynaptic test reflex could be observed The middle traces shows the resting activity during midbrain stimulation

elicited Note that whereas in precollicular cats locomotion can be induced this does not occur in the intercollicular preparations (Shik *et al* 1966 a b 1967)

During stimulation of the brainstem locomotor region these late discharges could also be evoked and in five preparations at a stimulation strength of the nerve that was not high enough to evoke a discharge prior to the stimulation as in Fig 3 In the test series of Fig 4 no discharge was evoked at 8 subsequent tests before stimulation whereas 8 tests performed during brainstem stimulation all evoked a discharge as in Fig 4 lower record (*i e* a statistically significant difference for sign test) More often discharges were evoked prior to brainstem stimulation which however increased in amplitude during stimulation These effects occurred with a stimulation strength of the brainstem which did not significantly alter the excitability of the α motoneurones Hence it is concluded that the neuronal network responsible for these discharge is directly influenced by the brainstem stimulation Ascending hindlimb forelimb reflexes are facilitated by locomotor region stimulation (Budakova and Shik 1970) but the discharges studied here have a duration and a latency which bears no resemblance to the spino bulbospinal effects of Shimamura and Livingston (1963) and are presumably due to a different neuronal network that either involves spinal as well as supraspinal relays or only a complex spinal interneuronal system It is of particular interest however that there is a striking resemblance between these discharges and those that are evoked in the low spinal cat after DOPA has been injected intravenously (Andén *et al* 1966 a Jankowska *et al* 1967 a)

Both occur with a latency of 100—200 ms and are increased progressively to a maximum after 1/2 a second to decline again (see Fig 4 lower record) Stimulation of ipsilateral peripheral nerves at high strength (from gr II III muscle afferents and cutaneous afferents) gives rise to activity in ipsilateral flexor motoneurones (filaments to tenuissimus (Fig 4) and semitendinosus) whereas stimulation of contra

lateral afferents gives rise to activity in extensors The longlasting discharge in flexors blocks the activity in extensors and vice versa There is thus a reciprocal organization Stimulation of an ipsilateral nerve can give rise first to a discharge in a flexor similar to Fig 4 with accompanying inhibition of extensor activity but subsequently the extensors can be activated and then again flexors These effects have also been observed in the present experiments as well as after DOPA (Jankowska *et al* 1967 a see Grillner 1969 a Fig 1) If the short train (3—5 pulses at 300 Hz) used for stimulation is prolonged successively to 100 ms or more the onset of the discharge is correspondingly delayed both after DOPA (Jankowska *et al* 1967 a) and in the present precollicular preparation Thus there appear to be many similarities between the late discharges that can be evoked after DOPA and in the precollicular cat whereas no differences have been demonstrated

Recall that the spinal neuronal network released after DOPA was postulated to generate alternate activation of flexors and extensors in locomotion (Jankowska *et al* 1967 a Lundberg 1969) and that spinal cats after DOPA in fact can walk (Grillner 1969 b Budakova 1971) In conclusion stimulation of the mesencephalic locomotor region releases the neuronal mechanism for locomotion and in parallel lowers the threshold for evoking similar discharges to the ones observed after DOPA Rhythmic activity can be observed in the filament if the forelimbs are moved as under locomotion during midbrain stimulation whereas midbrain stimulation in itself does not give rise to such activity The corresponding finding for the uncurarized preparation was reported by Shik *et al* (1966)

d) *Other effects evoked from the locomotor region*

Stimulation of the locomotor region increased the activity of efferents with small amplitude recorded in ventral root filaments as in the uncurarized preparation (Severin 1970) This activity can be assumed to be due to an increased activity in *γ efferents*

In 1 exp a dorsal root filament was mounted for recording of dorsal root potentials A train of pulses (9 pulses at 200 Hz 18 μA) in the locomotor region gave rise to no effect at a strength sufficient to evoke locomotion in the uncurarized preparation (18 μA at 30 Hz) If the strength was raised a dorsal root potential occurred at about 1 5 times threshold for locomotion From the same dorsal root filament a DRP from the sural nerve could be evoked

Discussion

Although stimulation of the very circumscribed region in the brainstem which we have referred to as the mesencephalic locomotor region gives rise to locomotion it cannot be taken for granted that effects evoked by electrical stimulation of this region necessarily are related to the generation of locomotion Let us nevertheless consider the effects evoked in relation to the expected requirements for l

An effective transmission in the shortlatency pathways from cutaneous and nociceptive afferents would seem disturbing for the locomotion in that it could allow a sudden inhibition of ipsilateral extensors, due to naturally occurring stimuli during walking and it thus seems appropriate that such reflex effects are depressed Correspondingly the inhibition via group II afferents from toe extensors to ankle extensors should be depressed whereas some caution must be used for extrapolating these effects to other muscles The linkage is minimally trisynaptic (Lundberg 1969 a) Since the direct excitability of the *a* motoneurones is not influenced the depression of the reflex transmission must be due either to postsynaptic inhibitory effects on the interneuronal level or to presynaptic inhibition on either afferent or on interneuronal terminals Although we tend to favour the possibility that the effects are exerted postsynaptically it should be recalled that a DRP can be evoked in unknown afferent fibers by stimulation of the locomotor region at a strength somewhat higher than for evoking locomotion It cannot be excluded that a moderate presynaptic depolarisation should not have been revealed with the recording at dorsal root level

In the Results section we pointed out the marked similarities between the long lasting discharges that can be evoked after DOPA and after precollicular decerebration (Fig 4) particularly during stimulation of the mesencephalic locomotor region *In the discussion below it will be assumed that these effects are evoked by the same spinal interneuronal network* that was described by Jankowska *et al* (1967 a b) This network is organized for an alternate activation of flexors and extensors and it has been suggested that it should provide a more or less differentiated central program for activation of muscles in the step cycle of the individual limb (Lundberg 1969 b) If it is taken into account that in both preparations in which locomotion can be induced experimentally (*i e* after DOPA and during midbrain stimulation) these late discharges occur *it is natural to relate the occurrence of late longlasting discharges and locomotion For the discussion below this will be our second assumption* It should be noted that whereas in acute spinal cats no locomotion can be induced it does occur in the chronic spinal preparation In this preparation such late discharges can be evoked (Lundberg and Vyklicky unpubl) in contrast to the acute spinal cat when this occurs only after DOPA

Although these late discharges occasionally can be evoked by stimulation of peripheral nerves including only gr II afferents (gr I are never effective) the effective volley must usually activate also fibres with smaller diameter (Jankowska *et al* 1967 a *cf* above) which might be activated in locomotion This seems at first sight very surprising but since the input to these centres seems very wide (Jankowska *et al* 1967 a Grillner 1969 b) one nerve represents only a small fraction of the total input It is likely that if this network is responsible for locomotion it receives also a more specific input for example from hip joint afferents as suggested by Shik and Orlovski (1965) The fact that these discharges can be evoked by a stimulus such as the one used here might merely be due to a fortunate coincidence rather than indicating a more specific mechanism

It is conceivable that this network requires a certain continuous input (*cf* Wilson 1964) may it be from peripheral or central structures, in order to raise the excitability level to a niveau in which the system can give rise to alternate activation of flexors and extensors For this argument it is relevant to discuss some results from a much more primitive vertebrate The dogfish seems unique in that it swims continuously after spinalization with well coordinated locomotion (Lissman 1946 a b) If the spinal dogfish is curarized the alternate activity of the segmental ventral roots disappears slowly with a decrease of the swimming frequency and eventually disappears altogether If however this curarized animal is *stroked on the side of the body*, inducing an exteroceptive inflow this suffices to reinstigate alternate activation of the two sides (B L Roberts 1969) A simple explanation would be to interpret this as an effect of increasing the excitability level of the responsible neuronal network

A somewhat similar finding is that of Budakova (1971), who in the precollicular cat (identical with that of the present experiments) has stimulated a lumbar dorsal root at low frequencies (2 Hz) which enables the cat to perform locomotion on the treadmill at frequencies which can have no relation to the dorsal root stimulus These effects disappear if the mesencephalic locomotor region including also a region somewhat ventral to the circumscribed region of Shik *et al* 1967 is destroyed or if an intercollicular section is made It should be recalled that peripheral nerve stimulation evokes late discharges in the precollicular cat which disappear with an intercollicular section (Results section)

These various findings would be explained if after DOPA an interneuronal network is released that in the acute spinal cat is entirely blocked and that this same spinal network is partially released in the precollicular cat but not to the extent that locomotion can be induced with a treadmill If however the dorsal roots are activated the activity in this system is enhanced or if the mesencephalic locomotor region is stimulated there is also an increased release of the same neuronal network allowing locomotion on the treadmill The ability to generate locomotion under these conditions is dependent upon the region in and below the mesencephalic locomotor region as well as upon structures in the rostroventral portion of the midbrain around the red nuclei (see Fig 1) The latter can be destroyed *without significant effects* on the induced locomotion (Shik *et al* 1967)

Which descending systems are responsible for the observed spinal effects

Among the great number of descending systems present rather few are known to give effects such as these evoked from the locomotor region in this curarized preparation Table I compares the effects of locomotor region stimulation studied in these experiments with the effects evoked from 3 descending systems which are known to give some of the encountered effects An injection of DOPA in the spinal cat has similar effects as locomotor region stimulation whereas 5 HTP iv in addition gives marked changes in α motoneurone excitability (Andén *et al* 1964 Ahlman *et al* 1971) the dorsolateral reticulospinal system on the other hand does not release the pathway for late long lasting discharges (Engberg *et al* 1968)

TABLE I

	Depr. of shortlat. effects	Effect on longlast. disch.	Incr. of mn. activ.	Excitabil. change in mn.
Locomotor reg. stim.	+	+	+	0
DOPA system	−	+	+	0
5-HTP system	+	−	+	+
Dorsolateral system	+	0	?	?

The effect of injections of DOPA and 5-HTP respectively has been shown to be related to the release of the transmitters noradrenaline and 5-HT respectively in the spinal cord (Andén *et al.* 1964, 1966 b) and it is most likely that DOPA i.v. in a spinal cat corresponds to an activation of the noradrenergic unmyelinated reticulo-spinal system and 5-HTP i.v. to an activation of descending 5-HT fibers from the raphe nuclei. It should be noted that DOPA and 5-HTP can produce all the effects listed. It cannot be taken for granted, however, that under natural conditions all these effects are evoked simultaneously by one system. Perhaps separate monoaminergic neurones elicite separate effects as *e.g.* if one type of neurones controls only the shortlatency transmission and another the release of late discharges.

In 3 preliminary expts. an i.v. injection of 5-HTP (50 mg/kg) *in a low spinal and decerebrate (unanesthetized)* cat only resulted in an excessive tonus in extensors sometimes changing to excessive prolonged flexor tonus. No sign of alternate activation of flexors and extensors bearing any resemblance to locomotion could be observed (Grillner and Shik, unpubl.). Note, however, that in the recent rabbit experiments of Buser and Viala (1971) they claim to have found alternate activation of flexor and extensor muscles that they relate to locomotion from both DOPA and 5-HTP i.v.

Hence a combined activation of parts of these descending systems could well produce the observed effects. Consider, however, that DOPA i.v. to a spinal cat can give rise to locomotion on a treadmill with an EMG pattern that for the main muscles does not differ from that of the intact cat although the gait does not usually appear altogether normal (Budakova 1971). For this reason *it seems more likely that the noradrenergic system is activated by stimulation of the mesencephalic locomotor region and that it is of paramount importance for changing the neuronal activity of the spinal cord to what is needed for generating the alternate activation of flexors in the step cycle of the individual limb.* It is important to test this hypothesis further experimentally by use of appropriate blocking agents. If this interpretation is correct, it provides the first example of a synaptic activation of the noradrenergic system and it should be recalled that although a variety of indirect evidence strongly support that DOPA acts through the release of noradrenaline from the terminals of these descending NA fibers, the effect of activation of these neurones has not yet been studied.

The effects encountered here can be revealed in the spinal cord without rhythmic activity and can be explained by an activation of the noradrenergic system It is most likely however, that other systems are also influenced Whereas slow monoaminergic systems can be used for changing some neuronal circuitry of fundamental importance for generating the basic locomotor pattern it is most likely that fast descending systems are also influenced (*e g* the efferent pathways of cerebellum) They can be used for rapid adjustments of the relative degree of activity of various muscles in the locomotor cycle It should be noted that neurones of these particular pathways *e g* the rubro vestibulo- and medial reticulospinal pathways can indeed be influenced from the locomotor region stimulation and more over they are phasically active in the locomotor cycle (Orlovski 1970 1972) In the decerebellate cat however these pathways are not phasically active during active locomotion None of these pathways are known to either inhibit the reflex transmission in the shortlatency pathways from the periphery or to release late discharges They influence *α* and or *γ* motoneurones either directly or via a few interneurones (Hongo *et al* 1969 Grillner and Lund 1968 Grillner and Hongo 1972)

In conclusion the depression of shortlatency transmission and the release of late discharges can be explained by one or some combination of the slow fiber systems discussed above but not by the fast descending systems The responsible fibers could switch on the spinal rhythm generating mechanism by its tonic effect on the spinal cord The most probable candidate seems to be the noradrenergic reticulospinal system Perhaps it is appropriate to end by stating that an important neuronal circuit as the stepping generator very likely is controlled not by one but by several systems many of which might still be unknown

The authors wish to thank prof V S Gourfinkel and A Lundberg for stimulating discussions and A S Jagodnitsyn for skilful technical assistance The experiments were carried out in Moscow while S G had a grant from the Royal Swedish Academy of Engin and were supported by the Swedish Med cal Research Council proj no B 14X 3026 and Petrus and Augusta Hedlunds stiftelse

References

AHLMAN H S GRILLNER and M UDO *The effect of 5-HTP on the static fusimotor activity* and the tonic stretch reflex on an extensor muscle *Brain Res* 1971 *27* 393—396

ANDEN N E M G M JUKES and A LUNDBERG Spinal reflexes and monoamine liberation *Nature* (Lond) 1964 *202* 1222—1223

ANDEN N E M G M JUKES and A LUNDBERG The effect of DOPA on the spinal cord 2 A pharmacological analysis *Acta physiol scand* 1966 b *67* 387—397

ANDEN N E M G M JUKES A LUNDBERG and L VYKLICKY The effect of DOPA on the spinal cord 1 Influence on transmission from primary afferents *Acta physiol scand* 1966 a *67* 373—386

BARD P and M B MACHT The behaviour of chronically decerebrate cats *Neurological Basis of Behaviour* Ciba Fond Symp Ed by N E W Wolstenholme and C M O Connor J & A Churchill Ltd London 1958 55—75

BERMAN A L *The brain stem of the cat* A cytoarchitectonic atlas with stereotaxic coordinates The Univ of Wisconsin Press 1968

BERNSTEIN N *The coordination and regulation of movements* Perg Press Oxford 1967

BROWN T G The intrinsic factors in the act of progression in the mammal *Proc Roy Soc B* 1911 *84* 308—319

BROWN T G On the nature of the fundamental activity of the nervous centres together with an analysis of the conditioning of rhythmic activity in progression and a theory of the evolution of function in the nervous system *J Physiol* (Lond) 1914 *48* 18—46

BROWN T G Unpublished cinematographic analysis on the stepping patterns of chronic high decerebrate cats Presentation to the *British Physiol Soc* 1941

BUDAKOVA N N Stepping movements evoked by a rhythmic stimulation of a dorsal root in mesencephalic cat *Fiziol Zh* (Mosk) 1971 *57* 1632—1640 (In russian)

BUDAKOVA N N and M L SHIK Effects produced by stimulation of the brainstem region evoking locomotion on the ascending reflexes in mesencephalic cats *Bull exp Biol Med* 1970 *72* No 1 7—11 (In russian)

BURKE R E JANKOWSKA and G TEN BRUGGENCATE A comparison of peripheral and rubrospinal synaptic input of triceps surae *J Physiol* (Lond) 1970 *207* 709—732

ECCLES R M and A LUNDBERG Supraspinal control of interneurones mediating spinal reflexes *J Physiol* (Lond) 1959 *147* 565—584

ENGBERG I and A LUNDBERG An electromyographic analysis of muscular activity in the hind limb of the cat during unrestrained locomotion *Acta physiol scand* 1969 *75* 614—630

ENGBERG I A LUNDBERG and R W RYALL Reticulospinal inhibition of transmission in reflex pathways *J Physiol* (Lond) 1968 *194* 201—223

GAMBARJAN P P G N ORLOVSKY T Y PROTOPOPOVA F V SEVERIN and M L SHIK The activity of muscles during different gaits and adaptive changes of moving organs in family Felidae Morphology and ecology of vertebrates *Proc Inst Zool Acad Sci USSR* 1971 *48* 220—239

GELFAND I M and M L TSETLIN *Mathematical modeling of mechanisms of the central nervous system in models of the structural functional organization of certain biological systems* Ed by I M Gelfand V S Gurfinkel S V Fomin and M L Tsetlin MIT Press Cambr Mass 1971 1—22 (Transl from russian, original publ 1966)

GRAY J The role of peripheral sense organs during locomotion in vertebrates *Physiological Mechanisms in Animal Behaviour* Symp Soc exp Biol No 4 Cambridge Univ Pres 1950 112—126

GRILLNER S The influence of DOPA on the static and the dynamic fusimotor activity to the triceps surae of the spinal cat *Acta physiol scand* 1969 a *77* 490—509

GRILLNER S Supraspinal and segmental control of static and dynamic γ motoneurones in the cat *Acta physiol scand* 1969 b Suppl *327* 1—34

GRILLNER S and T HONGO Vestibulospinal effects on motoneurones and interneurones in the lumbosacral cord *Basic Aspects of Central Vestibular Mechanisms* Ed by A Brodal and O Pompeiano Progress in Br Res (In press)

GRILLNER S and S LUND The origin of a descending pathway with monosynaptic action on flexor motoneurones *Acta physiol scand* 1968 *74* 274—284

HINSEY J C S W RANSON and R F MCNATTIN The role of the hypothalamus and mesencephalon in locomotion *Arch Neurol Psychiat* (Chic) 1930 *23* 1—43

HOLMQVIST B and A LUNDBERG Differential supraspinal control of synaptic actions evoked by volleys in the flexion reflex afferents in alpha motoneurones *Acta physiol scand* 1961 *54* Suppl 186 1—51

HOLST E VON Erregungsbildung und Erregungsleitung im Fischruckenmark *Pflugers Arch ges Physiol* 1935 *235* 345—359

HONGO T E JANKOWSKA and A LUNDBERG The rubrospinal tract I Effects on alphamotoneurones innervating hindlimb muscles in cats *Exp Brain Res* 1969 *7* 344—364

JANKOWSKA E M G M JUKES S LUND and A LUNDBERG The effect of DOPA on the spinal cord 5 Reciprocal organization of pathways transmitting excitatory action to alpha motoneurones of flexors and extensors *Acta physiol scand* 1967 a *70* 369—388

JANKOWSKA E M G M JUKES S LUND and A LUNDBERG The effect of DOPA on the spinal cord 6 Half-centre organization of interneurones transmitting effects from the flexor reflex afferents *Acta physiol scand* 1967 b *70* 389—402

KURO M Nervous control of micturition *Physiol Rev* 1965 *45* 425—494

LISSMAN M W The neurological basis of the locomotory rhythm in the spinal dogfish (scyllium canicula Acanthiasis vulgaris) I Reflex behaviour *J exp Biol* 1946 a *23* 143—161

LISSMAN M W The neurological basis of the locomotory rhythm in the spinal dogfish (scyllium canicula Acanthiasis vulgaris II The effect of deafferentation *J exp Biol* 1946 b *23* 162—176

LUNDBERG A Integration in the reflex pathway In *Muscular Afferents and Motor Control* Nobel Symposium I Ed by R Granit Almqvist & Wiksell Stockholm 1966 275—305

LUNDBERG A *Reflex control of stepping* The Nansen Memorial Lecture V Universitetsforlaget Oslo 1969 b

LUNDBERG A Convergence of excitatory and inhibitory action on interneurones in the spinal cord In *The Interneuron* Ed by M A B Brazier UCLA Forum Med Sci No 11 University of California Press Los Angeles 1969 a 231—265

ORLOVSKY G N Influence of the cerebellum on the reticulo pinal neurones during locomotion *Biophysics* 1970 *15 5* 928—936 (Eng transl)

ORLOVSKY G N Activity of vestibulo pinal neurones during locomotion *Brain Res* In press

ROAF H E and C S SHERRINGTON Further remarks on the mammalian spinal preparation *Quart J exp Physiol* 1910 *3* 209—211

ROBERTS B L Spontaneous rhythms in the motoneurones of spinal dogfish (Scylorhinus canicula) *J Mar Biol Ass U K* 1969 *49* 33—49

SEVERIN F V The role of the gamma motor system in the activation of the extensor alpha motoneurones during controlled locomotion *Biophysics 1970 15 6 1138—1145* (Eng transl)

SHERRINGTON C S Flexion reflex of the limb crossed extension reflex and reflex stepping and standing *J Physiol* (Lond) 1910 *40* 28—121

SHIMAMURA M and R B LIVINGSTON Longitudinal conduction systems serving spinal and brainstem coordination *J Neurophysiol* 1963 *26* 258—272

SHIK M L and G N ORLOVSKY Coordination of the limbs during running of the dog *Biophysics* 1965 *10* 1148—1159 (Eng transl)

SHIK M L G N ORLOVSKY and F V SEVERIN Organization of locomotor synergism *Biophysics* 1965 *10* 1148—1159 (Eng transl)

SHIK M L F V SEVERIN and G N ORLOVSKY Control of walking and running by means of electric stimulation of the midbrain *Biofizika* 1966 a *11* 756—763 (In russian)

SHIK M L F V SEVERIN and G N ORLOVSKY The brainstem structures responsible for the evoked locomotion *Fisiol of the USSR* 1967 *53* 1125—1132 (In russian)

SIROTA M G T I SIROTA and M L SHIK The circulation during the evoked locomotion of the mesencephalic cat *B ll exper b ol a medic* 1971 *72* Nr 2 3—6

SZEKELY G G CHEZ and G VOROS The activity pattern of limb muscles in freely moving normal and deafferented newts *Exp Brain Res* 1969 *9* 53—62

TSETLIN M L Issledovanija po teorii automatov i modelirovanjo biologitjeskix sistem Isdatelstvo Nauka Glavnaja redaktsija Fisiko-matematitjeskoj literatur Moscow 1969

VIALA D and P BUSER Modalites d obtention de rythmes locomoteurs chez le lapin sp nal par traitement pharmacologiques (DOPA 5 HTP d amphetam ne) *Brain Res* 1971 *35* 151—165

WILSON D M The origin of the flight motor command in grasshoppers In *Neuronal Theory and Modeling* Ed by R F Reiss Stanford Univ Press 1964 331—345

WOODS J W Behaviour of chron c decerebrate rats *J Neur physiol* 1964 *27* 535—541

Acta physiol scand 1973 87 334—343

From AB Draco* Research and Development Laboratories Lund Sweden

Dual Effects on the Sphincter of Oddi and Gallbladder Induced by Stimulation of the Right Great Splanchnic Nerve

By

CARL G A. PERSSON

Received 5 June 1972

Abstract

PERSSON C G A. *Dual effects on the sphincter of oddi and gallbladder induced by stimulation of the right great splanchnic nerve* Acta physiol scand 1973 87 334—343

The right great splanchnic nerve was stimulated in 15 anesthetized cats Effects on gallbladder pressure resistance to flow through the sphincter of Oddi and (in 6 cats) intramural duodenal pressure were evaluated. By nerve stimulation the sphincter was consistently contracted and the intestine relaxed The gallbladder showed variable slight responses except in 3 cats where consistent relaxation was obtained. Cholecystokinin (i v) relaxed the sphincter and contracted the gallbladder During the action of the hormone the nervous response was counteracted in the sphincter in the gallbladder relaxation was obtained in all cats. Noradrenaline (i v) mimicked the responses induced by adrenergic nerve stimulation The contraction of the sphincter induced by nerve stimulation and by noradrenaline was inhibited by phenoxybenzamine and atropine The relaxation of the gallbladder was prevented by propranolol. It is concluded that the adrenergic splanchnic nerves as well as i v noradrenaline mainly activated α receptors in the sphincter of Oddi (inducing contraction) and β receptors in the gallbladder (inducing relaxation) Results obtained with the nerve stimulation and the 3 blocking agents also confirmed the presence of contraction mediating atropine sensitive cholinoceptors in the sphincter and the gallbladder contraction mediating α receptors in the gallbladder and relaxation mediating β receptors in the sphincter

Stimulation of the right splanchnic nerve in the cat has been shown to produce relaxation of the gallbladder (Bainbridge and Dale 1905 Pallin and Skoglund 1964) Results obtained with tyramine noradrenaline and adrenaline on isolated gallbladder strips taken from normal and reserpinized cats support the view that the function of the adrenergic nerves is to relax the gallbladder (Persson 1972 a) Studies on the isolated sphincter of Oddi similar to those on the isolated gallbladder strips indicate that the adrenergic function is to contract the sphincter of Oddi (Persson 1971) This view is supported by the finding that noradrenaline contracts the cat sphincter of Oddi *in situ* (Liedberg and Persson 1970 Persson and Ekman 1972) Also the guinea pig sphincter has been shown to contract *in situ* when adrenaline

* Subsidiary to AB Astra Sweden

was given (Crema *et al* 1965) Crema and co-workers on the other hand report that noradrenaline as well as the stimulation of the right splanchnic nerve mainly produces relaxation of the terminal part of the bile duct in the cat (Benzi *et al* 1964 Crema *et al* 1964) In the canine sphincter of Oddi various responses to adrenaline are reviewed by Hallenbeck (1967) These observations leave the effect of splanchnic innervation of the sphincter of Oddi still open to question as also pointed out by Wyatt (1967)

It was thought of interest to study whether stimulation of adrenergic nerves could affect resistance to flow through the sphincter of Oddi and gallbladder pressure in a way corresponding to the adrenergic functions found in the *in vitro* studies (Persson 1971 1972 a) In the present study the effects on biliary motility in cat by stimulation of the right great splanchnic nerve were investigated The nervous responses were studied also during the action of cholecystokinin as the *in vitro* experiments suggested that adrenergic effects could counteract the dual mechanical responses in the sphincter and in the gallbladder induced by the hormone

Method

15 adult cats fasted for 16 h were anesthetized with 40 mg/kg of pentobarbital (Abbott) i p Additional doses of 6 mg/kg were given i v when needed The cats were placed on a heating table (37 C) and were given artificial respiration Blood pressure was measured by cannulation of the carotid artery The chest was opened on the right side and the right great splanchnic nerve was cleaned from surrounding tissue and placed over a pair of platinum electrodes which were ligated in position The nerve was protected with cotton wool soaked in liquid paraffin Usually the nerve was tied off proximal to the electrodes The abdomen was opened and the common bile duct was cannulated towards the duodenum and perfused with saline as described earlier (Persson and Ekman 1972) The perfusion rate was kept constant usually 1 65 ml/h by use of an infusion pump (Perfusor B Braun Melsungen 71 100) The cystic duct was ligated and a saline filled catheter with drainage hole was introduced through the fundus of the gallbladder and ligated with a purse string suture (*cf* Persson 1972 a) Care was taken that no bile escaped from the gallbladder in cats with a low bile content in the gallbladder some saline was infused To evaluate the effects on the intestine a piece of the femoral vein was taken and inserted into the intestinal wall about 2 cm distal to the sphincter of Oddi and perfused similar to the sphincter (For details of this experimental procedure see Persson and Ekman 1972) The positions of the different catheters are illustrated in Fig 1 The intramural pressure of the intestine as reflected by the perfused venous inserts was recorded in 6 cats Gallbladder pressure blood pressure and the perfusion pressures were recorded in all cats through pressure transducers (Statham AC 28P) on a Grass polygraph (7PI) The nerve was stimulated by square wave pulses from a Grass stimulator (S5) (frequency 10 Hz duration 7 ms voltage 5—8 V) Trains of pulses were applied for 10—20 s In [illegible] cats the experiment was interrupted and the adrenals were tied off

Drugs used cholecystokinin pancreozymin (CCK) (Prof J E Jorpes G I H Laboratories Karolinska Institutet Sweden) (—) noradrenaline bitartrate (Sigma Chemical Company USA) propranolol chloride (ICI Ltd England) phenoxybenzamine chloride (Smith Kline and French England) atropine sulphate (Pharmacopoea Nordica) acetylcholine chloride (Calbiochem USA) the C terminal octapeptide of cholecystokinin (C8-CCK) (obtained as a gift from Dr M A Ondetti The Squibb Institute for Medical Research New Brunswick USA) The drugs were given in the vena cava through a catheter introduced through the femoral vein

Results

Spontaneous pressure changes were recorded by perfusion of the sphincter of Oddi (Fig 2—6) With the low perfusion rate used the rythmic pressure variations closely resembled those recorded when the sphincter had been isolated and was

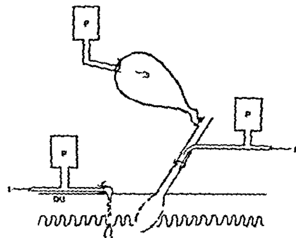


Fig 1 *Experimental set up for recording of intra gallbladder pressure through a saline filled open tip catheter (upper left) resistance to flow through the perfused sphincter of Oddi (right) intramural duodenal pressure through the perfused venous insert (lower left) P = pressure transducer I = infusion of saline DU = duodenum.*

perfused *in vitro* (Persson 1971 1972 b) When no drug affected the resistance to flow through the sphincter a pressure range of 8—25 cm H_2O in the common duct catheters was recorded

In all 15 cats stimulation of the right great splanchnic nerve increased the resistance to flow through the sphincter of Oddi (Fig 2—6) An increase in resistance to flow has previously been shown to correspond to a contraction of the sphincter muscle (Persson 1971 1972 b) this response will be referred to as contraction in the following Two cats showed variable responses but mostly contractions were obtained Another cat responded to nerve stimulation only after treatment with the β adrenoreceptor blocking agent propanolol (1 mg/kg) when the sphincter was consistently contracted Except for these 3 cats stimulation of the right splanchnic regularly caused an increase in resistance to flow through the sphincter This response was obtained also after adrenalectomy

10 of the cats were given atropine 0 5—1 mg/kg In this dose atropine blocked the effect of acetylcholine 1—5 μg/kg which otherwise produced strong contraction of the sphincter Atropine either did not affect (Fig 3) or transiently depressed the spontaneous sphincter activity which returned when the effect of acetylcholine was still abolished In most cats atropine counteracted part of the contraction induced by nerve stimulation Two of the cats had to be given propranolol 1 mg/kg to show a clear-cut contractile response also after atropine However in some cases when large pressure variations were recorded by perfusion of the sphincter the contraction induced by nerve stimulation was more marked after atropine because then the spontaneous activity was depressed by the action of this drug 11 of the cats were treated with the α adrenoceptor blocking agent phenoxybenzamine (0 5—5 mg/kg) which alone or together with atropine abolished the increase in resistance to flow induced by stimulation of the splanchnic nerve (Fig 4 5) In 1 expt a pronounced relaxation of the sphincter resistance by nerve stimulation was seen after treatment with the α receptor blocker This relaxation was prevented by propranolol (1

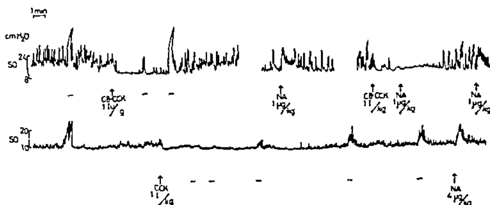


Fig 2 Effects in 2 cats (above and below) on sphincter of Oddi (SO) induced by right splanchnic nerve stimulation (—) C8 CCK CCK and noradrenaline (NA) C8-CCK and CCK inhibit the spontaneous sphincter activity and counteract the contractions induced by nerve excitation and by i v noradrenaline The gradual recovery of the nervous effect after CCK is illustrated

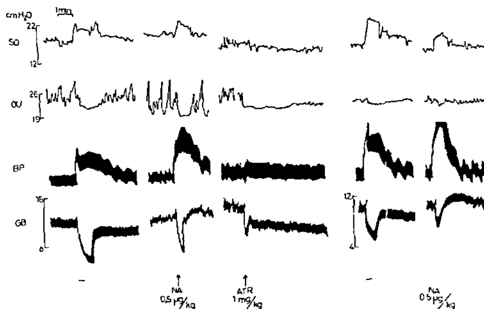


Fig 3 Effects on sphincter of Oddi (SO) duodenum (DU) arterial blood pressure (BP) and gallbladder (GB) induced by stimulation of the right great splanchnic nerve (—) and by i v noradrenaline (NA) The sphincter is contracted while the duodenum and the gallbladder are relaxed Atropine relaxes duodenum and gallbladder but only slightly affects the sphincter Atropine does not seem to affect the nervous response or the effect of noradrenaline on the sphincter but the effects on the intestine and the gallbladder are somewhat less pronounced when atropine has depressed these organs In this cat cholecystokinin was not needed to obtain relaxation in the gallbladder

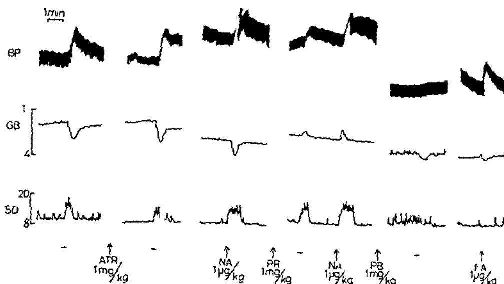


Fig 4 Effects before and after atropine (ATR) propanolol (PR) and phenoxybenzamine (PB) on arterial blood pressure (BP) gallbladder (GB) and sphincter of Oddi (SO) induced by stimulation of the right great splanchnic nerve (—) and by i v noradrenaline (NA) The sphincter is contracted also after atropine and propranolol but not after phenoxybenzamine The gallbladder is relaxed but after propranolol it is contracted This contraction is present after atropine but abolished by phenoxybenzamine In this cat cholecystokinin was not needed to obtain relaxation in the gallbladder

mg/kg) Propranolol did not counteract the contraction induced by the adrenergic nerve stimulation Instead in some cats it unmasked the splanchnic induced contraction as noted above As reported earlier (Liedberg and Persson 1970) propranolol occasionally increased the resistance to flow through the sphincter This response was present also after treatment with atropine and phenoxybenzamine

Noradrenaline (1—4 μg/kg) produced a contraction of the sphincter similar to that of the adrenergic nerve stimulation (Fig 2—4) The effect of noradrenaline was prevented by phenoxybenzamine (Fig 4)

Cholecystokinin (CCK) and the C terminal octapeptide (C8 CCK) relaxed the sphincter and inhibited the spontaneous pressure variations (Fig 2 5 6) The effect of C8 CCK was of shorter duration than that of CCK (cf Fig 2) This hormonal relaxation counteracted the response to nerve stimulation and the effect of i v noradrenaline to a varying degree (Fig 2 6) In one cat a given dose of CCK partly counteracted the nervous response but abolished it when the cat was pretreated with atropine The mechanical activity of the intestine as recorded by the perfused venous graft was only partly parallel to the sphincter activity Intestinal responses were recorded in only 6 cats in one of these the perfused venous insert yielded spontaneous pressure changes too variable to allow evaluation In the others stimulation of the right splanchnic relaxed the intestine to a varying degree The relaxation occurred in parallel with the contraction in the sphincter (Fig 3 5)

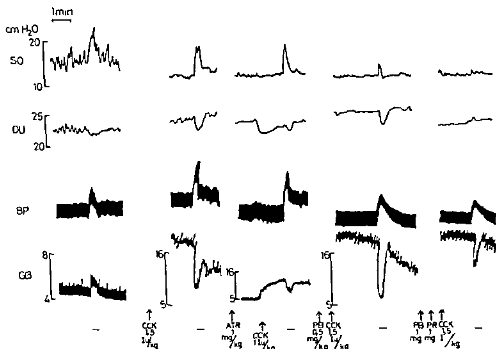


Fig 5 Effects on sphincter of Oddi (SO) duodenum (DU) arterial blood preessure (BP) and gallbladder (GB) induced by stimulation of the right splanchnic nerve (—) before and after cholecystokinin (CCK) atropine (ATR) phenoxybenzamine (PB) and propanolol (PR) The sphincter is contracted This response is present after atropine but is abolished by phenoxybenzamine The duodenum is relaxed This response is inhibited by phenoxybenzamine plus propranolol The gallbladder is relaxed only after CCK. This response is somewhat increased after phenoxybenzamine but markedly counteracted by propranolol CCK relaxed the sphincter and the duodenym but contracted the gallbladder CCK in the given doses only slightly counteracted the nervous response in the sphincter The nerve was excited after maximum effect by CCK.

Noradrenaline also relaxed the intestine at the same time as it contracted the sphincter (Fig 3) In some cases the relaxation to noradrenaline was followed by a contraction CCK and C8 CCK usually relaxed the intestine as shown earlier by means of this method (Persson and Ekman 1972)

The recordings from the gallbladder revealed no spontaneous activity but respiration movements were continuously seen The resting gallbladder pressure in the cats varied between 4—12 cm H O In 3 of the cats the resting pressure was consistently decreased when the right splanchnic was stimulated (Fig 3 4) The usual response to nerve stimulation was otherwise slight increases of pressure sometimes combined with slight relaxation of the gallbladder (Fig 5) Noradrenaline (1—4 μg/kg) produced similar responses (Fig 3 4) However when the gallbladder pressure had been increased by treatment with CCK or C8-CCK a relaxation could be obtained in all cats when the adrenergic nerves were excited This includes the cats which

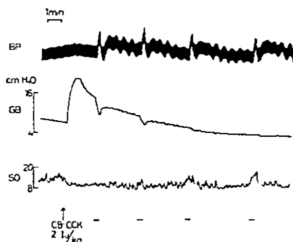


Fig 6 Effects on arterial blood pressure (BP) gallbladder (GB) and sphincteer of Oddi (SO) induced by excitation of the right great splanchnic nerve (—) The curve illustrates the gradual decrease in effect on the gall bladder along with the gradual increase in effect on the sphincter by nerve stimulation when the effects of the C terminal octapeptide of cholecystokinin (C8-CCK) in the 2 organs are declining

besides CCK also had to be given atropine (1 case) or phenoxybenzamine (2 cases) before a clear cut relaxation was obtained Relaxation of the gallbladder was obtained also after adrenalectomy

Atropine usually decreased the gallbladder pressure (Fig 3) and blocked the contracting effect of acetylcholine Noradrenaline relaxed the gallbladder as did the nerve stimulation However in some experiments the noradrenaline produced relaxation was followed by a transient increase in pressure (Fig 3) Propranolol (1—2 mg/kg) was given to 8 cats The β receptor blocker prevented the relaxing adrenergic responses (Fig 4 5) In 2 cats stimulation of the splanchnic nerve invariably caused contraction of the gallbladder after propranolol pretreatment This ntraction was not affected by atropine but was abolished by phenoxybenzamine (Fig 4) All the actions of CCK and C8 CCK seemed to be unaffected by propranolol phenoxybenzamine and atropine

Discussion

The splanchnic nerves are the main source of sympathetic innervation to the upper abdominal viscera in the cat (Hirt 1934) Baumgarten and Lange (1969) have shown that adrenergic nerves probably containing noradrenaline are present *in the* gallbladder and the sphincter of Oddi of the cat Stimulation of the right great splanchnic nerve and administration of noradrenaline would accordingly provide information about a possible sympathetic control of biliary motility

The results of the present investigation showed that stimulation of the right great splanchnic nerve in the cat increased the resistance to flow through the sphincter of Oddi and decreased the gallbladder pressure when it had been elevated by CCK or C8-CCK Noradrenaline was shown to produce qualitatively similar effects as the nerve stimulation These findings are in accordance with the adrenergic functions suggested by *in vitro* studies with gallbladder and sphincter of Oddi preparations (Persson 1971 1972 a)

The excitatory effect of the right splanchnic nerve on resistance to flow through the sphincter could as shown in some cats be partly prevented by atropine It cannot be excluded that the adrenergic nerves contain cholinergic fibres (*cf* H Persson 1971) and a stimulation of these might have caused the atropine sensitive part of the contraction The whole contractile response or that which resisted treat ment with atropine was blocked by the α receptor blocking agent phenoxybenzamine which also abolished the excitation induced by noradrenaline Consequently it seems that stimulation of the adrenergic splanchnic nerves mainly activated α adrenoceptors in the phincter thereby contracting the sphincter After α receptor blockade it was clearly seen in one case that the nerve stimulation caused relaxation of the sphincter The relaxation was blocked by propranolol and was probably a β receptor mediated effect as it has been shown earlier that the β receptor function is to relax the sphincter (*cf* Liedberg and Persson 1970 Persson 1971) The finding that propranolol unmasked a nerve mediated contraction of the sphincter in 2 of the cats that had received atropine agrees with this view

In contrast to the present findings Crema and co-workers (Crema *et al* 1964 Benzi *et al* 1964) reported that the resistance to flow through the cat sphincter of Oddi was mainly decreased both by stimulation of the right great splanchnic nerve and by noradrenaline These authors measured the flow through the sphincter by means of a drop counter (3—6 drops/min) This method does not allow a continuous recording of the sphincter resistance However this is possible when pressure changes are recorded during constant rate perfusion Furthermore the rates of flow through the sphincter reported by Crema and co workers were 4—16 times higher than those used in the present study A high flow rate would extend the sphincter lumen and thus increase the possibility that duodenum which is relaxed by adrenergic activity may influence the flow resistance through the sphincter Methodological differences may thus have contributed to the conflicting results

Earlier it was shown that the perfused venous insert for intestinal recording of intramural pressure changes is a more sensitive method than recording from an open tip catheter placed in the intestinal lumen It was also suggested that it reflects duodenal activity of possible importance for the resistance to flow through the sphincter of Oddi (Persson and Ekman 1972) The main response to noradrenaline and to stimulation of the right splanchnic was relaxation of the intestine along with the contraction induced in the sphincter of Oddi This agrees with the view that the adrenergic effect is relaxation of the intestine (Daniel 1968) and shows that the sphincter muscle reacts differently to the surrounding duodenum (*cf* Liedberg and Persson 1970 Persson 1971 Persson and Ekman 1972)

Stimulation of the right splanchnic nerve as well as i v noradrenaline decreased the C8 CCK or CCK raised gallbladder pressure This finding agrees with the results reported by Pallin and Skoglund (1964) They found that prolonged stimula tion of the right splanchnic nerves decreased the pressure re ponse to subsequent doses of CCK Bainbridge and Dale (1905) recorded consistent sympathetic relaxa tion of the gallbladder before the hormone CCK had been discovered These authors

however had raised the pressure in the gallbladder with the aid of an intraluminal balloon It thus seems that the gallbladder pressure must be elevated before a clear cut relaxation can be obtained by stimulation of the sympathetic nerve supply to the gallbladder Bainbridge and Dale (1905) also demonstrated contractions of the gallbladder when they stimulated the right splanchnic nerve but were able to show that this effect was due to a swelling of the liver Such a swelling may have been responsible for the pressure increase seen in the present study in gallbladders that had not been contracted by CCK However other factors may also have contributed to contraction of the gallbladder induced by stimulation of the right splanchnic nerve In 1 case relaxation of the CCK treated gallbladder by splanchnic stimulation could be obtained only after the cat had been given atropine and 2 cats showed clear relaxations only after they had been given an α receptor blocking agent These results suggested that α adrenoceptors and atropine sensitive cholinoceptors could mediate contraction of the gallbladder This possibility is supported by the contractions obtained in isolated gallbladder strips by acetylcholine and when the β receptors had been blocked by noradrenaline (Persson 1972 a) A contraction induced by acetylcholine and similarly acting compounds has been reported by many investigators as reviewed by Hallenbeck (1967)

The β receptor blocking agent propranolol prevented the inhibitory nervous effect in the gallbladder and in two cases reversed the response The excitatory effect in the gallbladder after β receptor blockade was abolished by α receptor blockade a finding that supports the view that α receptors can mediate contraction in the gallbladder (*cf* Persson 1972 a) Propranolol alone occasionally increased the resistance to flow through the sphincter This effect did not seem to be caused by its eceptor blocking property as it occurred also after atropine and phenoxybenz ine

It is concluded that by stimulation of the adrenergic right great splanchnic nerves mainly α receptors in the sphincter of Oddi and β receptors in the gallbladder are activated Thereby the sphincter its contracted and the gallbladder is relaxed These effects might be of significance during the filling phase and promote the entry of bile into the gallbladder The adrenergic effects are contrary to the actions of CCK or C8 CCK in these organs These findings show that the effects of splanchnic innervation counteract hormonal effects on biliary motility

The technical assi tance of Mrs M Ekman and Mrs I Erjefalt is gratefully acknowledged

References

BAINBRIDGE F A and H H DALE The contractile mechanism of the gallbladder and its extrinsic nervous control *J Physiol* (Lond) 1905—1906 *33* 138—155

BAUMGARTEN H G and W LANGE Extrinsic adrenergic innervation of the extrahepatic biliary duct system in guinea pigs cats and rhesus monkeys *Z Zellforsch* 1969 *100* 606—615

BENZI G F BERTE A CREMA and G FRIGO Actions of sympathomimetic drugs on the smooth muscle at the junction of the bile duct and duodenum studied *in situ* *Brit J Pharmacol* 1964 *23* 101—114

CREMA A G BENZI G M FRIGO and F BERTE Occurrence of α and β receptors in the bile duct *Proc Soc exp Biol* (N Y) 1965 *120* 158—160

CREMA A F BERTE G BENZI and G M FRIGO The responses of the sphincterial areas of the extrahepatic biliary tract to the stimulation of sympathetic and parasympathetic nerves *Acta physiol lat amer* 1964 *14* 24—32

DANIEL, E E In *Handbook of Physiology* Alimentary Canal (Ed W Heidel Williams and Wilkins Co Baltimore) 1968 *4* 2267—2324

HALLENBECK G A In *Handbook of Physiology* Alimentary Canal (Ed W Heidel Williams and Wilkins Co Baltimore) 1967 *2* 1007—1026

HIRT A *Handbuch der vergleichenden Anatomie der Wirbeltiere* L Bolk E Goppert E Kallius W Lubosch Berlin 1934 *2* 735—776

LIEDBERG G and C G A PERSSON Adrenoceptors in the cat choledochoduodenal junction studied *in situ* *Brit J Pharmacol* 1970 *39* 619—626

PALLIN B and S SKOGLUND Neural and humoral control of the gallbladder emptying mechanism in the cat *Acta physiol scand* 1964 *60* 358—362

PERSSON C G A Adrenoceptor functions in the cat choledochoduodenal junction *in vitro* *Brit J Pharmacol* 1971 *42* 447—461

PERSSON C G A Adrenoceptors in the gallbladder *Acta pharmacol* (Kbh) 1972 a *31* 177—185

PERSSON C G A Resistance to flow through the pancreatic duct by the isolated cat sphincter of Oddi *Experientia* (Basel) 1972 b *28* 276—278

PERSSON C G A and M EKMAN Effect of morphine cholecystokinin and sympathomimetics on sphincter of Oddi and intramural pressure in cat duodenum *Scand J Gastroent* 1972 *7* 345—351

PERSSON H *Studies on regulatory mechanisms of the peripheral autonomic nervous system with special regard to adrenoceptor functions* Thesis Sweden 1971

WYATT A P *The relationship of the sphincter of Oddi to the stomach duodenum and gall bladder* *J Physiol* (Lond) 1967 *193* 225—232

Acta physiol scand 1973 87 344—347

From the Department of Anatomy University of Helsinki Finland

Effect of Prolonged Nerve Blockage on the Development of the Myoneural Junction

By

J JUNTUNEN and H TERÄVAINEN

Received 6 June 1972

Abstract

JUNTUNEN J and H TERÄVAINEN *Effect of prolonged nerve blockage on the development of the myoneural junction* Acta physiol scand 1973 87 344—347

The electrical conductivity of the sciatic nerve was locally blocked by repeatedly injecting long acting local anesthetic (Marcaine® adrenaline) dorsally to the collum femoris of new born rat for 7 days Structural alterations of the postsynaptic muscle fibre membrane occurring during the development were followed with a light microscope after the activity of myoneural acetylcholinesterase (E C 3 1 1 7) enzyme was histochemically demonstrated Unlike anatomical denervation by cutting the sciatic nerve nerve blockage with the anesthetic did not prevent structural development of the myoneural junction from occurring as in normal untreated muscles The observations would seem to indicate that chemical agents other than acetylcholine are responsible for the regulatory effect of the nerve on the structural alterations of the postsynaptic part of the muscle fiber membrane

Motoneurone controls the structure metabolic activities and electrical properties of striated muscle fibres (Buller *et al* 1960 Diamond and Miledi 1962 Hogan *et al* 1965 Cutman 1967 Robert and Oester 1970) including alterations in the post synaptic muscle membrane of the myoneural junction during postnatal development (Teravainen and Juntunen 1968) In a newborn rat the myoneural junction consists of the apposition of the axon terminals to the unfolded straight membrane of the muscle fiber The postsynaptic infoldings typical of the myoneural junctions of adult animals appear at the age of about one week (Teravainen 1968) The adult shape is attained at the age of about one month

The purpose of the present work was to test whether uninterrupted physiological liberation of acetylcholine by the nerve endings in the form of intact impulse transmission is responsible for the induction of the structural alterations of the post synaptic muscle membrane

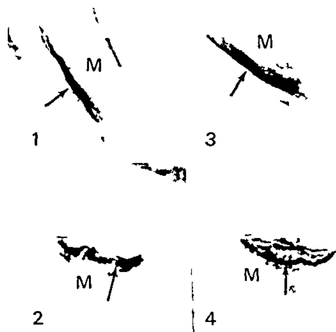


Fig 1 Side view of precipitate due to the local acetylcholinesterase activity of two-day old rat tibialis anterior muscle demonstrating myoneural junction (arrow) M postsynaptic muscle fiber The precipitate demonstrates a straight postsynaptic membrane devoid of infoldings typical of the more mature junctions × 900

Fig 2 Acetylcholinesterase activity at a myoneural junction (side view) in the tibialis anterior muscle after injecting Marcaine® adrenaline into the sciatic nerve for 7 days The precipitate has a somewhat cup-like appearance due to partial invagination of the axon terminals into the postsynaptic muscle fiber (M) In addition short infoldings of the membrane are present (arrow) × 900

Fig 3 Myoneural acetylcholinesterase in tibialis anterior muscle denervated by ipsilateral transsection of the sciatic nerve 7 days prior to incubation The structure of the postsynaptic membrane (arrow) is essentially the same as it was prior to the operation (Fig 1) M postsynaptic muscle fiber × 900

Fig 4 Myoneural acetylcholinesterase activity in the tibialis anterior muscle after injections of physiological saline adrenaline into the sciatic nerve for 7 days The structure (arrow) is similar to the junctions after Marcaine® adrenaline treatment (Fig 2) M postsynaptic muscle fiber × 900

Material and Methods

Marcaine® adrenaline 0 5 % (1 200 000) (1 n butyl DL piperidine 2 carboxylic acid 2 6-dimethylanilide AB Bofors Nobelkrut, Sweden) was injected perineurally to the left sciatic nerve of one day old rats in the region of the collum femoris In the beginning the dosage was 0 03 ml increasing gradually to 0 05 ml It was close to DL o since 9 out of 27 died during repeated injections This was done in order to obtain the maximal conduction block. Other possible effects of Marcaine® adrenaline on the myoneural development were excluded by proper controls The mean duration of the nerve blockage should be between 1—12 h (mean 10 4) according to the manufacturer This was clinically verified 15 of the analyzed animals were injected every 12th h and 3 every 8th h There were no differencies in the myoneural development between these two groups Pieces were taken from the tibialis anterior muscle of

one of the animals injected every 8th h for electron microscopy in order to check the presence of intact myoneural junction These were found indicating that the injections performed did not have a denervating effect The sciatic nerve of 3 animals was transsected with scissors at the level of the collum femoris to control the structural development of the postsynaptic membrane separated anatomically from the motoneurone. The normal development was studied in 3 untreated animals from the same litters and from the contralateral side of the animals injected with physiological saline together with the adrenaline 1 200 000)

The histochemical reactions were made from the formalin calcium fixed tibialis anterior muscle using free floating 30 μm thick sections Acetylcholinesterase (AChE E C 3 1 1 7) was demonstrated with the Gomori (1952) version of the thiocholine technique The substrate (acetylthiocholine iodide Fluka AC Buchs) was added after preincubation for 30 min with 10^{-5} M of the inhibitor tetra isopropylpyrophosphoramide (iso OMPA L Light & Co Ltd Colnbrook) to exclude the activity of other cholinesterases (E C 3 1 1 8) The precipitate due to AChE activity was made visible with sodium sulphide immersion at pH about 5 0 For details of the method see Teravainen (1968)

Results and Discussion

Fig 1 shows the distribution of the AChE activity of the myoneural junction at the beginning of the experiment The postsynaptic membrane is straight There is no sign of invagination of the synaptic axon terminals into the muscle fiber nor are post synaptic infoldings visible After seven days of injections of Marcaine® adrenaline the myoneural structure is cup like Postsynaptic infoldings are demonstrated by the distribution of the reaction product (Fig 2)

In contrast to the Marcaine® adrenaline treatment nerve transsection causes a standstill in the development of the postsynaptic muscle membrane There was no sign of postsynaptic infoldings 7 days after the operation (Fig 3) On the other hand the postsynaptic membrane of the saline adrenaline injected side and that of the non treated animals (Fig 4) are comparable to the structure of the myoneural junctions innervated by the Marcaine® adrenaline treated sciatic nerve

The above results demonstrate that blockage of the impulse transmission from the motor nerve for seven days does not cause cessation of the structural development of the postsynaptic part of the muscle membrane at the same time However the anatomical connection must at the same time be intact indicating that some presumably chemical substance which is not likely to be acetylcholine (ACh) is needed for continuous postsynaptic development The present observations on the inductive action of the motoneurone in the absence of nerve impulses are compatible with physiological observations of the lack of spontaneous fibrillation of the muscle fibers during prolonged conduction block (Robert and Oester 1970) Even if the nerve conduction blockage is likely to cause marked reduction in the total quanta ACh released from the nerve ending it does not prevent the spontaneous quantal release of ACh (mepp) (Lomo and Rosenthal 1972)

References

BULLER A J J C ECCLES and R M ECCLES Interactions between motoneurones and muscles in respect of the characteristic speeds of their responses J Physiol (Lond) 1960 150 417—439

DIAMOND J and R MILEDI A study of foetal and newborn rat muscle fibers J Physiol (Lond.) 1962 162 393 —408

GOMORI G (ed) *Microscopic histochemistry principles and practice* p 137—221 Chicago Chicago University Press 1952

GUTMAN E Neurotropic relations in the regeneration process In *Progress in brain research* vol XV p 72—112 (eds M Singer and J P Schade) Amsterdam—London—New York Elsevier 1967

HOGAN G L D M DAWSON and F C A ROMANUL Enzymatic changes in denervated muscle II Biochemical studies *Arch Neurol* (Chic) 1965 *3* 274—282

LOMO T and J ROSENTHAL Control of acetylcholine sensitivity by muscle activity in the rat *J Physiol* (Lond) 1972 *221* 493—513

ROBERT E D and Y T OESTER Nerve impulses and trophic effect Absence of fibrillation after prolonged and reversible conduction block *Arch Neurol* (Chic) 1970 *22* 57—63

TERAVAINEN H Carboxylic esterases in developing myoneural junctions of rat striated muscle *Histochemie* 1968 *10* 307—315

TERAVAINEN H and J JUNTUNEN Effect of temporary denervation on the development of the acetylcholinesterase positive structures of the rat myoneural junction. *Histochemie* 1968 *15* 261—269

Acta physiol scand 1973 87 348—358

From the Department of Pharmacology, School of Medicine, Linkoping Sweden

Relationship between Cyclic AMP, Phosphodiesterase Activity, Calcium and Contraction in Intestinal Smooth Muscle

By

ROLF G G ANDERSSON

Received 7 June 1972

Abstract

ANDERSSON R G G *Relationship between cyclic AMP phosphodiesterase activity calcium and contraction in intestinal smooth muscle* Acta physiol scand 1973 87 348—358

Effects of contracting agents on the mechanical and the metabolic activity in smooth muscle have been studied The contraction induced by carbacholine in colon muscle from rabbit was preceded by a decrease of the cyclic AMP content After development of the tension the level of cyclic AMP was increased the phosphodiesterase activity was reduced and the phosphorylase *a* activity and glycogenolysis were stimulated These effects were blocked by atropine but not by an adrenergic *β* blocking agent In the Ca poor muscle there was a sustained decrease the cyclic AMP content by carbacholine Contraction induced by K ions showed similar abolic actions as carbacholine and these effects were Ca dependent too The contracting effect of imidazole was preceded by an activation of phosphodiesterase and by reduction of cyclic AMP The metabolic effects induced by imidazole were not Ca dependent Imidazole potentiated the contracting action of carbacholine and K Ca in the same concentrations as those activating the contractile system inhibited the phosphodiesterase activity in homogenate of colon muscle The relationship between cyclic AMP Ca and contraction in smooth muscle is discussed

There are strong indications that in skeletal and cardiac muscles contraction and relaxation are dependent on an increase and decrease respectively of the concentration of free Ca ions in the smooth muscle cell (review Sandow 1965 Ebashi and Endo 1968) and there is mounting evidence that Ca has the same function in smooth muscle (Edman and Schild 1961 Daniel 1963 Hurwitz von Hagen Joiner 1967 Ruegg 1971)

The glycogenolysis following contraction in skeletal muscle is at least partly dependent on an increased formation of phosphorylase *a* (Posner Stern and Krebs 1965), but changes in the concentration of metabolites (AMP ATP and G-6 P) may be of importance for an increased phosphorylase *b* activity (Morgan and Parmeggiani 1964) In contracting skeletal muscle the increased formation of phosphorylase *a* was not accompanied by an increase in cyclic AMP (Posner et al

1965) but has been ascribed to a stimulation of the activity of phosphorylase *b* kinase by Ca^{++} (Walsh *et al* 1970)

In smooth muscle from rabbit colon the relaxing action mediated by adrenergic β receptors was associated with an increased content of cyclic AMP and enhanced activity of phosphorylase *a* and stimulation of glycogenolysis These effects were present in a Ca^{++} poor preparation too In the normal but not in the Ca^{++} poor preparation both isoprenaline and cyclic AMP reduced the ATP and CrP contents (Andersson 1972 a)

Contraction of smooth muscle was accompanied by an activation of phosphorylase *a* (Mohme Lundholm 1962 Brody and Diamond 1967) increased glycogenolysis and increased formation of lactate (Lundholm and Mohme Lundholm 1957 1962) and a reduction of the ATP and CrP contents (Beviz *et al* 1965) Thus there is a surprising similarity between the metabolic actions observed during contraction and relaxation of smooth muscle

In an earlier study it was demonstrated that removal of Ca^{++} reduced the cyclic AMP content and the phosphorylase *a* activating of smooth muscle from rabbit colon On readdition of Ca the cyclic AMP content and phosphorylase *a* activity increased again (Andersson 1972 a)

In this study the relation between contraction changes in the cyclic AMP content phosphorylase *a* activation and the dependence of these actions on the presence of Ca^{++} has been further investigated in smooth muscle In contrast to skeletal muscle the phosphorylase *a* activation in contracting smooth muscle was found to be associated with an increased cyclic AMP content This increase was in contrast to that accompanying adrenergic β receptor stimulation Ca dependent and associated with an inhibition of the phosphodiesterase activity A preliminary report of some of these results has been presented (Andersson Lundholm and Mohme Lundholm 1972)

Methods

Rabbits weighing 2—3 kg were used They were stunned and exsanguinated Pieces of the colon muscle freed from mucosa were suspended in Krebs-Henseleit bicarbonate buffer solution at 37 C aerated with 95 % O_2 + 5 % CO_2 The muscles were attached to plastic holders as described by Lundholm and Mohme Lundholm (1966) and the isometric contractions were recorded by an FT 03 force transducers on a Grass polygraph The length and tension of the preparations were standardized as described earlier (Andersson and Mohme Lundholm 1969 1970)

To reduce the calcium content of the preparations they were suspended in a calcium free buffer to which EDTA in a concentration of 0 2 mM had been added the buffer was changed several times After 60 min the preparation was incubated in Ca^{++} free Krebs buffer solution but without EDTA Mg^{++} (1 2 mM) was present in a normal concentration At this time the muscle had no tension of its own and did not respond to contracting agents

When the concentration of metabolites was to be determined the muscles were frozen at −80 C in frigen 12 containing solid CO_2 The phopshorylase activity was determined according to Brading *et al* (1962) Hexose phosphates ATP and CrP were assayed as described by Andersson and Mohme Lundholm (1970) Cyclic AMP was determined according to Kakiuchi and Rall (1968) The phosphodiesterase activity was measured according to Poch (1971) Ca^{++} was determined from HNO_3 digested samples by a Unicam Atom Spectrophotometer (Parker 1964)

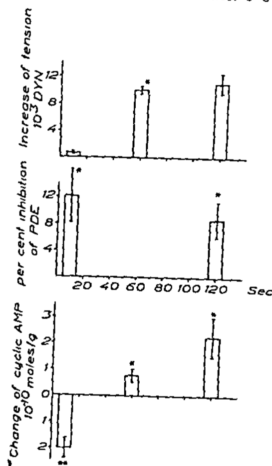


Fig 1

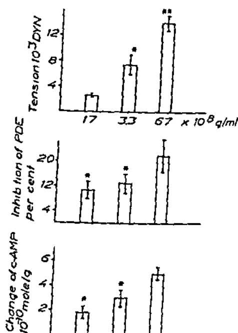


Fig 2

Fig 1 Dose re ponse relationship of the action of carbacholine on mechanical and metabolic events in rabbit colon muscle Changes of tension phosphodiesterase activity and cyclic AMP content from control values 60 s after addition of the drug Mean ± S E ($n = 6$—8) Statistical significance in paired tests from the same muscle tissue is denoted by * = $p < 0\ 05$ ** = $p < 0\ 01$ *** = $p < 0\ 001$

Fig 2 Time response relationship of the action of carbacholine (3×10^{8} g/ml) on tension, phosphodiesterase activity and cyclic AMP content of rabbit colon muscle The mean control value of cyclic AMP = $7\ 7 \pm 1\ 6 \times 10^{10}$ mol/g Mean ± S F ($n = 8$) Statistical significance as in Fig 1

Results

Metabolic and mechanical effects of carbacholine in normal and Ca poor rabbit colon

When the cyclic AMP content was determined 60 s after the addition of carbacholine there was a significant increase (Fig 1) There was a correlation between the increase in tension and cyclic AMP content and also between these parameters and the inhibition of the phosphodiesterase activity after carbacholine in different concentrations (1 7—6 7 × 10^{8} g/ml) (Fig 1)

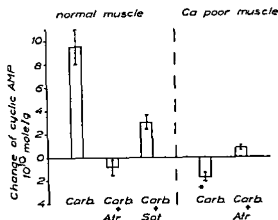


Fig 3 Influence of carbacholine (1 7× 10 g/ml) alone or in combination with atropine (8 3×10 7 g/ml) or sotalol (1 2×10 5 g/ml) on cyclic AMP content of normal and Ca poor rabbit colon muscle 60 s after addition of carbacholine Mean±S E (n = 8—10) Statistical s gnificance as in Fig 1

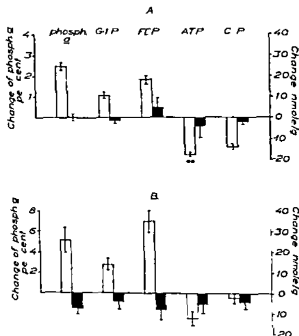


Fig 4 Influence of 1 7×10 7 g/ml carbacholine ((1 A) and 128 mEq/l K ions (1 B) on phosphorylase a activity in per cent of total phosphorylase activity content of gluco e 1 phosphate (G 1 P) fructose 1 6 d phosphate (FDP) ATP and CrP in nmol/g Open bars represent changes from control values in normal muscle filed bars changes from control value in Ca⁺⁺ poor muscle Mean ±S E (n = 6—8) Statistical sign ficance as in Fig 1

When the time response relationship between tension and cyclic AMP content wa studied (Fig 2) it was found that just before the tension started to rise—after 10 s—there was a decrease of the cyclic AMP content—despite an inhibition of the phosphodiesterase activity The cyclic AMP content had increased after 60 and 120 s (Fig 2)

The possibility that carbacholine had released noradrenaline from sympathetic neurones in the muscle by a nicotinic action and thereby stimulated adrenergic

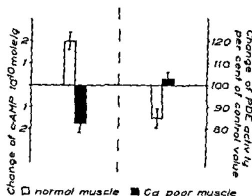


Fig 5

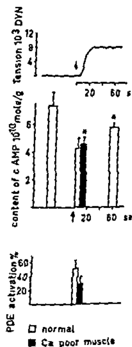


Fig 6

Fig 5 The action of K ions (128 mEq/l) on the cyclic AMP content and phosphodiesterase activity of normal or Ca poor colon muscle 60 s after its addition Mean±S E (n = 6—8) Statistical significance as in Fig 1

Fig 6 Influence of imidazole (2 5×10⁻⁴ g/ml) on tension cyclic AMP content and phosphodiesterase activity 10 or 60 s after its addition to normal or Ca⁺⁺ poor colon muscle The arrow indicate addition of imidazole Mean±S E (n = 6—8) * denotes the statistical significance of the changes from the control values

receptors was considered After treatment of the preparation with the adrenergic β receptor blocking agent sotalol—which in a concentration of 1 2×10 g/ml totally inhibited the cyclic AMP increasing actions of isoprenaline (Andersson 1972 a)—the cyclic AMP increasing action of carbacholine still persisted although it was reduced to some degree (Fig 3) The effect of carbacholine on the cyclic AMP content was blocked by atropine in a concentration of 8 3×10 g/ml (Fig 3)

In the Ca poor muscle preparation the total Ca content was reduced from 5 9±0 5×10⁻³ M to 1 51±0 20×10⁻³ M (Andersson 1972 a) Carbacholine had no increasing action on the cyclic AMP content or tension after 60 s in the Ca⁺⁺ poor muscle instead there was a decrease of the content of the cyclic nucleotide an effect blocked by atropine (Fig 3) The inhibition action of carbacholine on the phosphodiesterase activity was also eliminated in the Ca poor muscle The control value of phosphodiesterase was 27±0 3 nmol cyclic AMP hydrolysed/mg tissue/h in this muscle the corresponding value after carbacholine treatment was 27±0 5 In an earlier paper it was demonstrated that adrenergic β receptor stimulation in

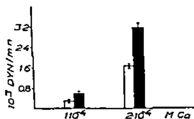


Fig 7

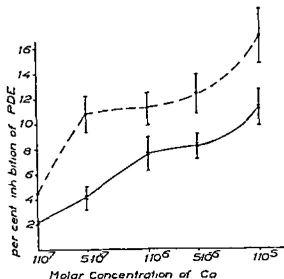


Fig 8

Fig 7 Influence of Ca on tension development per minute in Ca poor carbacholine treated colon muscle before (open bars) and after (filled bars) addition of imidazole (2.5×10^{-4} g/ml) Mean±S E (n = 6) The statistical significance bet een preparations with and with out imidazole is shown

Fig 8 Relationship between the pho phodiesterase activity of a homogenate from rabbit colon muscle and the concentration of free Ca^{++} ions in the homogenate The degree of inhibition is calculated in per cent of the activity of the same homogenate where the concentration of free Ca was reduced to $< 1\times10^{-7}$ M by addition of EGTA The activity was determined at a cyclic AMP concentration of 1×10^{-6} M ( ) and of 1×10^{-4} M (×———×) Mean± S E (n = 6)

creased the cyclic AMP content in the Ca poor preparation (Andersson 1972 a) In normal muscle carbacholine (1.7×10 g/ml) increased besides the tension the phosphorylase *a* activity and the contents of glucose 1 phosphate and fructose 1 6 diphosphate and reduced the ATP and CrP contents 60 s after its addition Carbacholine failed to produce any of these metabolic effects in the Ca^{++} poor preparation (Fig 4 A)

Influence of K ions and imidazole on the cyclic AMP content and metabolism during contraction It was of interest to find out whether other contracting agents had the same metabolic actions as carbacholine From Fig 5 is seen that contraction induced by addition of 128 mEq/l of K after 60 s had increased the cyclic AMP content stimulated the carbohydrate metabolism and reduced the ATP content (Fig 4 B) Further the pho phodiesterase activity was inhibited (Fig 5) In the Ca poor preparation these metabolic effects were blocked (Fig 4 B 5) and the cyclic AMP content (Fig 5) and phosphorylase *a* activity were reduced instead (Fig 4 B)

Imidazole ($2\ 5 \times 10^{4}$ g/ml) a wellknown activator of phosphodiesterase (Butcher and Sutherland 1962) increased the phosphodiesterase activity of rabbit colon, reduced the cyclic AMP content and contracted the muscle The reduction of the nucleotide was most marked after 10 s (Fig 6) The effect on the cyclic AMP content and phosphodiesterase activity was still present in the Ca poor preparation (Fig 6)

When a Ca poor muscle was suspended in a solution to which carbacholine ($1\ 7 \times 10$ g/ml) had been added to increase the Ca permeability of the membrane, the muscle contracted when Ca (2×10^{4} M) was added to the solution The contraction was markedly potentiated both regarding rate of tension development (Fig 7) and maximal tension when the muscle had been pretreated with imidazole Imidazole potentiated the contracting action of K too

Influence of Ca on the phosphodiesterase activity of smooth muscle homogenate The observed inhibition of the phosphodiesterase activity in the presence of Ca indicated that the concentration of free Ca in the myoplasm might be one regulator of the phosphodiesterase activity Cheung (1967) observed that Ca among other divalent ions (Cu and Zn), inhibited the activity of phosphodiesterase in an added concentration of 2×10^{3} M

Even Ca poor muscle contained appreciable amounts of calcium ($1\ 5 \pm 0\ 2 \times 10^{3}$ M) Methods for determination of free Ca in the myoplasm or in a homogenate are under discussion To obtain fixed concentrations of free Ca in the incubation solutions predetermined amounts of Ca and EGTA were added and the concentration of free Ca was calculated from the values of Ebashi and Endo (1968) whose calculations had given some higher values of free Ca than those of Schwarzenbach *al* (1957) EGTA chelates Mg to a rather small degree so the observed effects were not due to a variation in the Mg concentration

It has been reported (Beavo Hardman and Sutherland 1970 Thompson and Appleman 1971) that in some tissues (heart adipose tissue brain) 2 forms of phosphodiesterase exist with different K_m and V_{max} for cyclic AMP The activity of phosphodiesterase was therefore tested at 2 different concentrations of cyclic AMP (1×10^{4} and 1×10^{6} M) and different concentrations of Ca From Fig 8 it is seen that an inhibition of the activity was evident already at a calculated free Ca^{++} concentration of 5×10^{7} M and that the inhibition increased with elevating Ca concentrations The phosphodiesterase activity measured at the lower cyclic AMP concentration was inhibited to a greater degree than that measured at the higher concentration which might indicate that it was predominately the phosphodiesterase form with a low K_m for cyclic AMP that was inhibited

Discussion

Both carbacholine and K ions had a biphasic action on the cyclic AMP content of intestinal smooth muscle As an early action (after 10 s) and just as the contraction

started there was a decrease of the cyclic AMP content (Fig 2) although the phosphodiesterase activity was reduced (Fig 2) In the normal preparation carbacholine increased the cyclic AMP at 60 s at which time there was still a decrease in the Ca^{++} poor muscle (Fig 3) The phosphodiesterase activity was reduced in the normal but not in the Ca^{++} poor muscle The decrease of cyclic AMP was blocked by atropine This indicates that the production of cyclic AMP was reduced *i e* that the effect was probably dependent on a decrease in the adenyl cyclase activity This suggestion is in line with the results of Murad *et al* (1962) who demonstrated that acetylcholine reduced the adenyl cyclase activity of a homo genate from heart muscle In the Ca^{++} poor preparation K reduced the cyclic AMP content too (Fig 5) Whether this effect is a direct action of K ions on the smooth muscle or a result of an increased acetylcholine release from parasympathetic neurones in the muscles—a potassium effect demonstrated by Paton and Aboo Zar (1968)—remains to be elucidated

A biphasic action on the cyclic AMP was demonstrated too after having con tracted the vascular smooth muscle by stimulation of adrenergic *a* receptors The increase in cyclic AMP was Ca dependent and combined with a reduction of the phosphodiesterase activity (Andersson 1972 b)

In a homogenate of smooth muscle Ca ions in a concentration that activates the contractile system inhibited the phosphodiesterase activity (Fig 7) It therefore seems probable that the increase in cyclic AMP during contraction by carbacholine *and K was at least partly dependent on an inhibition of the phosphodiesterase* activity The possibility that the contracting agents had influenced the adenyl cyclase activity via Ca^{++} might be taken in consideration too

The effect of carbacholine on the cyclic AMP content was probably the result of a balance of a direct decreasing action on adenyl cyclase and a reduction of the phosphodiesterase activity by Ca^{++} An interesting question is if the changes of these enzyme activities was identical in all cell compartments or if local differences in the concentration of cyclic AMP arose There is some evidence of compartmentilization of cyclic AMP in smooth muscle (Andersson *et al* 1971)

Imidazole contracted the muscle and reduced the cyclic AMP content and was able to potentiate the contractile effect of carbacholine and K ions The mechanical and metabolic effects of imidazole were similar to those of cholecystokinin on gallbladder (Andersson *et al* 1972) It is thus evident that the contractile process could be dissociated from an increased cyclic AMP content Cyclic AMP probably counteracted the increase in tension an effect which was evident in vascular smooth muscle (Andersson 1972 b) The increased cyclic AMP content following contrac tion of smooth muscle is probably a kind of negative feed back mechanism which regulates the activating effect of Ca^{++} ions on the contractile process with regard to time and extent (Andersson Lundholm and Mohme Lundholm 1972)

Robison Butcher and Sutherland (1967) discussed the possibility that an increase in the cyclic AMP content produced relaxation and a decreased contraction of smooth muscle The present results are not in line with such a general statement

Imidazole ($2\,5\times10^{4}$ g/ml) a wellknown activator of phosphodiesterase (Butcher and Sutherland 1962) increased the phosphodiesterase activity of rabbit colon reduced the cyclic AMP content and contracted the muscle The reduction of the nucleotide was most marked after 10 s (Fig 6) The effect on the cyclic AMP content and phosphodiesterase activity was still present in the Ca poor preparation (Fig 6)

When a Ca^{++} poor muscle was suspended in a solution to which carbacholine ($1\,7\times10$ g/ml) had been added to increase the Ca permeability of the membrane the muscle contracted when Ca^{++} (2×10^{4} M) was added to the solution The contraction was markedly potentiated both regarding rate of tension development (Fig 7) and maximal tension when the muscle had been pretreated with imidazole Imidazole potentiated the contracting action of K too

Influence of Ca on the phosphodiesterase activity of smooth muscle homogenate The observed inhibition of the phosphodiesterase activity in the presence of Ca^{++} indicated that the concentration of free Ca in the myoplasm might be one regulator of the phosphodiesterase activity Cheung (1967) observed that Ca among other divalent ions (Cu and Zn) inhibited the activity of phosphodiesterase in an added concentration of 2×10^{3} M

Even Ca poor muscle contained appreciable amounts of calcium ($1\,5\pm0\,2\times10^{3}$ M) Methods for determination of free Ca in the myoplasm or in a homogenate are under discussion To obtain fixed concentrations of free Ca in the incubation solutions predetermined amounts of Ca and EGTA were added and the concentration of free Ca was calculated from the values of Ebashi and Endo (1968) whose calculations had given some higher values of free Ca^{++} than those of Schwarzenbach *et al* (1957) ECTA chelates Mg to a rather small degree so the observed effects were not due to a variation in the Mg^{++} concentration

It has been reported (Beavo Hardman and Sutherland 1970 Thompson and Appleman 1971) that in some tissues (heart adipose tissue brain) 2 forms of phosphodiesterase exist with different K_m and V_{max} for cyclic AMP The activity of phosphodiesterase was therefore tested at 2 different concentrations of cyclic AMP (1×10^{4} and 1×10^{6} M) and different concentrations of Ca From Fig 8 it is seen that an inhibition of the activity was evident already at a calculated free Ca^{++} concentration of 5×10 M and that the inhibition increased with elevating Ca^{++} concentrations The phosphodiesterase activity measured at the lower cyclic AMP concentration was inhibited to a greater degree than that measured at the higher concentration which might indicate that it was predominately the phosphodiesterase form with a low K_m for cyclic AMP that was inhibited

Discussion

Both carbacholine and K ions had a biphasic action on the cyclic AMP content of intestinal smooth muscle As an early action (after 10 s) and just as the contraction

ANDERSSON R and E MOHME LUNDHOLM Studies on the relaxing actions mediated by stimulation of adrenergic α and β receptors in taenia coli of the rabbit and guinea pig *Acta physiol scand* 1969 *77* 372—384

ANDERSSON R and E MOHME LUNDHOLM Metabolic actions in intestinal smooth muscle associated with relaxation mediated by adrenergic α and β receptors *Acta physiol scand* 1970 *79* 244—261

ANDERSSON R and K NILSSON Relaxation in intestinal smooth muscle role of cyclic AMP and calcium *Nature* (Lond) 1972 *238* 119—120

ANDERSSON K E R, ANDERSSON and P HEDNER Cholecystokinetic effect and concentration of cyclic AMP in gallbladder muscle in vitro *Acta physiol scand* 1972 *85* 511—516

ANDERSSON R L LUNDHOLM E MOHME LUNDHOLM and K NILSSON Role of cyclic AMP and Ca^{++} in metabolic and mechanical events in smooth muscle In *Advances in cyclic nucleotide research* Raven Press 1972

ANDERSSON R E MOHME LUNDHOLM N SVEDMYR and N VAMOS Relaxing and metabolic actions of ACTH in rabbit colon *Acta physiol scand* 1971 *81* 11—17

BEAVO J A J G HARDMAN and E W SUTHERLAND Hydrolysis of cyclic guanosine and adenosine 3 5 monophosphates by rat and bovine tissues *J biol Chem* 1970 *245* 5649—5655

BEVIZ A L LUNDHOLM E MOHME LUNDHOLM and N VAMOS Hydrolysis of adenosine triphosphate and creatine phosphate on isometric contraction of vascular smooth muscle *Acta physiol scand* 1965 *65* 268—272

BRODY T M and J DIAMOND Blockade of the biochemical correlates of contraction and relaxation in uterine and intestinal smooth muscle *Ann N Y Acad Sci* 1967 *137* 772—781

BUEDING E E BULBRING H KURIYAMA and G GERCKEN Lack of activation of phosphorylase by adrenaline during its physiological effect on intestinal smooth muscle *Nature* (Lond) 1962 *196* 944—946

BUTCHER R W and E W SUTHERLAND Adenosine 3 5 phosphate in biological materials I Purification and properties of cyclic 3 5 nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3 5 phosphate in human urine *J biol Chem* 1962 *237* 1244—1250

CHEUNG W Y Properties of cyclic 3 5 nucleotide phosphodiesterase from rat brain *Biochemistry* 1967 *6* 1079—1087

DANIEL E E Potassium movements in rat uterus studies in vitro II Effects of metabolic inhibitors ouabain and altered potassium concentrations *Canad J Biochem* 1963 *41* 2085

EBASHI S and M ENDO Calcium ion and muscle contraction *Progr Biophys molec Biol* 1968 *18* 123—183

EDMAN K A P and H O SCHILD Interaction of acetylcholine calcium and depolarization in the contraction of the smooth muscle *Nature* (Lond) 1961 *190* 350—352

HARDMAN J Stimulation of rat liver phosphodiesterase by cyclic GMP alteration by triton Ca^{++} and EGTA In *Abstract Int Conf on Physiol and Pharmacol of Cyclic AMP* Milan 1971 p 26

HURWITZ L P D JOINER and S VON HAGEN Mechanical responses of intestinal smooth muscle in a calcium free medium *Proc Soc exp Biol* (N Y) 1967 *125* 518—522

KAKIUCHI S and T W RALL The influence of chemical agents on the accumulation of adenosine 3 5 phosphate in slices of rabbit cerebellum *Molec Pharmacol* 1968 *4* 367—378

KAKIUCHI S and R YAMAZAKI Calcium dependent phosphodiesterase activity and its activating factor (PAF) from brain *Biochem biophys Res Commun* 1970 *41* 1104—1110

LUNDHOLM L and E MOHME LUNDHOLM The effect of adrenaline on the glycogen metabolism of smooth muscle *Acta physiol scand* 1957 *38* 237—254

LUNDHOLM L and E MOHME LUNDHOLM Studies on the effects of drugs upon the lactic acid metabolism and contraction of vascular smooth muscle *Acta physiol scand* 1962 *55* 45—63

LUNDHOLM L and E MOHME LUNDHOLM Length of inactivated contractile elements length tension diagram active state and tone of vascular smooth muscle *Acta physiol scand* 1966 *68* 347—359

MOHME LUNDHOLM E Phosphorylase activity of smooth muscle *Acta physiol scand* 1962 *54* 200—208

MONN E and R O CHRISTIANSEN Adenosine 3 5 monophosphate phosphodiesterase multiple molecular forms *Science* 1971 *173* 540—541

MORGAN H E and A PARMEGGIANI Regulation of glycogenolysis in muscle III Control of muscle glycogen phosphorylase activity *J biol Chem* 1964 *239* 2440—2445

MURAD F Y M CHI T W RALL and E W SUTHERLAND Adenyl cyclase III The effect of catecholamines and choline esters on the formation of adeno ine 3 5 phosphate by preparations from cardiac muscle and liver *J biol Chem* 1962 237 1233—1239

PARKER H E Magnesium calcium and zinc in animal nutrition *Atomic Absorption News letter* 1964 *13* 1—7

PATON W D M and M ABOO ZAR The origin of acetylcholine released from guinea pig intestine and longitudinal muscle strips *J Physiol* (Lond) 1968 *194* 13—33

POCH G Assay of phosphodiesterase with radioactively labelled cyclic 3 5 AMP as substrate *Naunyn Schmiedebergs Arch exp Path Pharmak* 1971 *268* 272—299

POSNER J B R STERN and E G KREBS Effects of electrical stimulation and epinephrine on muscle phosphorylase phosphorylase *b* kinase and adenosine 3 5 phosphate *J biol Chem* 1965 *240* 982—985

ROBISON G A R W BUTCHER and E W SUTHERLAND Adenylcyclase as an adrenergic receptor *Ann N Y Acad Sci* 1967 *139* 703—723

RUEGG J C Smooth muscle tone *Physiol Rev* 1971 *51* 201—248

SANDOW A Excitation contraction coupling in skeletal muscle *Pharmacol Rev* 1965 *17* 265—320

SCHWARZENBACH G H SENN and G ANDEREGG Komplexone XXIX Ein grosser Chelateffekt besonderer Art *Helv chim Acta* 1957 *XI* 1886—1900

THOMPSON W J and M M APPLEMAN Multiple cyclic nucleotide phosphodiesterase activities from rat brain *Biochemistry* 1971 *10* 311—316

UZONOV P and B WEISS Separation and characterization of multiple adenosine 3 5 monophosphate phosphodiesterase activities of rat brain *Fed Proc* 1972 *31* II 514

WALSH D A E G KREBS E M REIMANN M A BROSTRÖM J D CORBIN J P HICKENBOTTOM T R SODERLING and J P PERKINS The receptor protein for cyclic AMP in the control of glycogenolysis In *Role of cyclic AMP in cell function* (P Greengard and E Costa eds) Raven Press N Y 1970 265—285

Acta physiol scand 1973 87 359—367
From the Department of Physiology University of Goteborg Sweden

Interaction between the Fastigial Pressor Response and the Defence Reaction

By

BJORN LISANDER and JAN MARTNER

Received 10 June 1972

Abstract

LISANDER B and J MARTNER *Interaction between the fastigial pressor response and the defence reaction* Acta physiol scand 1973 87 359—367

Experiments were performed on chloralosed cats with recording of blood pressure heart rate and regional blood flows Electrical stimulation of the fastigial nucleus produced a pressor response During stimulation of the hypothalamic defence area fastigial stimulation decreased the muscle vasodilation and augmented the concomitant vasoconstriction in the skin and intestinal beds These effects from fastigial stimulation could be accounted for by activation of α adrenergic mechanisms However a fastigial inhibition of hypothalamically induced cholinergic vasodilation was also observed in the innervated cross circulated limb in which adrenergic blocking drugs had been administered This suggests that fastigial stimulation can interfere with the activation of the dilator fibres at some level within the central nervous system as well as in the periphery Both defence area and fastigial stimulation can also cause an adrenomedullary activation The defence reaction may include a weak activation of the adrenergic outflow to the muscles which is not readily apparent because of the baroceptor activation secondary to the widespread systemic adrenergic activation

The present investigation was prompted by the finding that stimulation of the cerebellar cortex can inhibit several components of the hypothalamically induced defence reaction including the cholinergic muscle vasodilation (Lisander and Martner 1971) The hypothesis was put forward that the fastigial nuclei may exert a general facilitatory influence on the defence reaction and that this influence in turn may be suppressed by Purkinje fibres emanating from the cerebellar cortex This mechanism would be in consonance with the finding that fastigial stimulation may induce sham rage in decorticate cats (Zanchetti and Zoccolini 1954) and also with the view that Purkinje cells can monosynaptically inhibit neurons within the fastigial nuclei (see Eccles Ito and Szentagothai 1967)

Stimulation of parts of the feline fastigial nucleus can induce a marked blood pressure rise (Miura and Reis 1970 Achari and Downman 1970) The present investigation has been devoted to a study of the interaction between this fastigial pressor response and the hypothalamically induced defence reaction Special attention has been paid to the possibility of a fastigial influence upon sympathetic cholinergic dilator fibre activity

Methods

Experiments were performed on 45 cats of both sexes. After induction with ether light anesthesia was maintained by i.v. administration of chloralose 30—50 mg/kg b.w. For stereotaxic stimulation the animal's head was fixed in a Horsley—Clarke apparatus. Following trephination and gentle removal of parts of the tentorium cerebelli sharp stainless steel monopolar electrodes were inserted vertically into the fastigial nuclei using Horsley-Clarke coordinates. Square wave pulses were delivered by a stimulator allowing for constant current stimulation at intensities of 0.05—0.4 mA and durations of, in most cases, 1 ms at a wide range of frequencies. Hypothalamic defence area stimulation was performed with an identical stimulator at the same current intensities and at durations of 1—2 ms and frequencies of 10—100 Hz. (The constant current stimulators were constructed by civ. ing. Lars Stage.) After each experiment anodal current of 1 mA was applied to the stimulation points for 30 s and the head of the animal was perfused by saline followed by formaldehyde. Stimulated parts of the central nervous system were paraffin embedded, sectioned and stained by the Nissl technique.

The vagi were dissected in the neck and placed on loose ligatures so that they could be cut in the course of the experiment. For baroceptor stimulation the carotid sinus region was gently dissected, partly isolated and connected to one of the femoral arteries by a tube. This tube could deliver constant mean levels of a pulsating pressure to the carotid sinus by means of a sigma motor pump, or it could transmit the ambient arterial systemic blood pressure of the animal. The sinus pressure was measured *via* a side branch of the tube connection by a P 23 AC transducer and recorded on a Grass polygraph. In some experiments, in addition, the outflow from the sinus was conducted to the external jugular vein *via* a tube system with an adjustable resistance so that mean sinus pressure could be adjusted to different levels at a constant flow pumped through the sinus region. The contralateral sinus nerve was freed and sectioned.

Heparin was given as an anticoagulant. Blood pressure was measured through a catheter in one femoral or brachial artery connected to a Statham P 23 AC transducer. Heart rate was recorded by connecting the pressure recording amplifier to a Grass tachograph unit. Muscle blood flow was measured as the outflow from the deep femoral vein, the paw circulation being excluded by a tight ligature at the ankle. After passing a closed optical drop recorder device operating an ordinate writer the blood was returned to the animal *via* one of the femoral veins. In most experiments, however, the leg was skinned and the calf muscles completely isolated, a hole being drilled in the femur and the bone marrow plugged.

Intestinal or cutaneous blood flow was recorded with the same technique. When intestinal lood flow was to be measured, the intestines with exception for a 20—30 g jejunal segment ere extirpated after which the venous outflow from the remaining segment was passed through drop recorder and returned to the animal *via* the portal vein. Skin blood flow in the hind aw was similarly recorded from the great saphenous vein, other major veins being ligated. ...an arterial blood pressure in the vascular bed studied was in some experiments kept constant by a screw clamp around the abdominal aorta. Arterial blood pressure was then followed both in the brachial and femoral arteries.—When cross circulation of a skeletal muscle vascular bed was to be performed, the femoral arteries and veins of the two animals were connected in such a way that the donor supplied the circulation of an isolated calf preparation. The sciatic nerve formed the only intact connection between the calf and the recipient. The arterial pressure of the donor was recorded from a side branch of the arterial tube and venous outflow from the calf was measured by a drop recorder coupled in series with the venous tubing connecting the calf with the donor. In a few experiments the adrenals were exposed transabdominally and the right one was ligated while the left one was denervated. However, in the majority of the experiments in which cross circulation was not performed, the time course of the cardiovascular responses was used as a criterion for their direct neurogenic origin. The hormonal responses were delayed at least 10 s.

Some of the animals received gallamine triethiodide (Flaxedil®, May and Baker, 2—4 mg/kg) to eliminate such cardiovascular effects that could be induced secondarily to somato-motor changes. Constant artificial respiration was then maintained by means of a respiration pump. Atropine (atropine sulphate) was given at 0.5—1 mg/kg b.w. and eserine (physostigmine salicylate) at 0.1 mg/kg b.w. i.v. The antiadrenergic drug guanethidine (Ismelin®, CIBA) was used at 2—4 mg/kg b.w. i.v. For β-adrenergic blockade alprenolol (Aptin®, Hässle, 0.5 mg/kg b.w. i.v.) or propranolol (Inderal®, I.C.I., 0.5 mg/kg b.w. i.v.) were given. Phentolamine (Regitin®, CIBA, 1 mg/kg b.w. i.v.) or dibenzyline (Smith, Kline and French, 2 mg/kg b.w. i.v.) were used to block α-adrenergic vascular effects. Noradrenaline (nor-adrenaline bitartrate) was injected i.v. in a saline solution at 1 μg/ml prepared just prior to use.—Some of the drugs were instead injected i.a. into the isolated calf muscle preparation at the same doses per kg tissue mass (estimated under the assumption that its weight is 1.7 per cent of that of the cat, Kjellmer 1964).

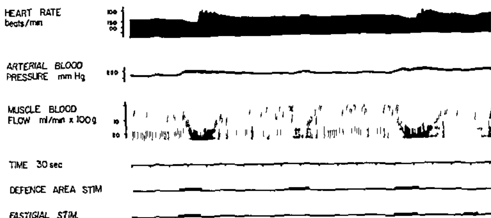


Fig 1 Cat 2.5 kg Adrenergic fibres blocked by guanethidine 3 mg/kg b.w. i.v. Effects of defence area stimulation (40 Hz 1 ms 0.2 mA) with and without concomitant cerebellar stimulation Note the greatly augmented muscle vasodilation as a response to defence area stimulation when the fastigial nucleus is simultaneously stimulated at 50 Hz 1 ms and 0.2 mA

Results

Stimulation within the rostral pole of the fastigial nucleus regularly increased arterial blood pressure heart rate and muscle blood flow resistance In addition to increases in blood pressure and heart rate defence area stimulation caused a marked skeletal muscle vasodilation which could be blocked almost completely by atropine When these two stimulations were performed simultaneously in cats not given atropine there was a muscle vasoconstriction of a magnitude similar to that seen when the fastigial nucleus alone was stimulated This was the case even at fastigial stimulation rates as low as 10 or occasionally at 5 Hz Below this frequency of fastigial stimulation however the profound atropine sensitive vasodilation observed when hypothalamic stimulation was performed alone could again be induced *The latter* stimulation usually raised mean arterial blood pressure only moderately but could markedly potentiate the pressure rise from a simultaneous fastigial stimulation Some fastigial stimulations had no significative influence on skeletal muscle flow resistance although a few of them caused a blood pressure rise These stimulations as well often completely or partly inhibited the muscle vasodilation induced by defence area stimulation

In six cats with intact adrenal glands given guanethidine (2—4 mg/kg i.v.) 3 of which were also given phentolamine (1 mg/kg i.v.) fastigial stimulations did not significatively influence skeletal muscle flow resistance *If such a fastigial stimulation* was performed simultaneously with a weak defence area stimulation (which reduced muscle flow resistance only by 25 %) the muscle resistance fell as much as 80 % with a latency of 15—20 s (Fig 1) The delayed peak dilation from these combined stimulations was hardly affected by atropine but strongly by the β adrenergic blockers

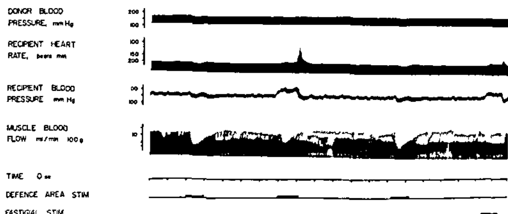
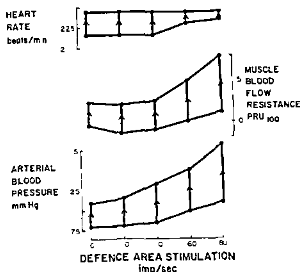


Fig 2 Cross-circulation experiment (donor 3 7 kg recipient 4 2 kg) recipient with intact vagi donor paralysed by gallamine and artificially ventilated The calf of the recipient is cross circulated and with innervation intact Guanethidine 3 mg/kg b w has been given i v to the donor Defence area stimulation (80 Hz 1 ms 5 V) gives a dilation in the vascular bed but the latter is unaffected by fastigial stimulation When the two stimulations are performed together however the muscle vasodilation is decreased by 25 per cent For further explanations see text

alprenolol and propranolol (0 5—1 mg/kg i v) suggesting that it was due to catecholamines released from the adrenal medulla After the β blocker combined stimulation never gave a dilation larger than that from hypothalamic stimulation alone

In 12 other expts the isolated calf was cross circulated from another animal The sciatic nerve was left intact and the patency of the autonomic innervation could be checked by carotid occlusion or gentle pulling of the carotid arteries eliciting vasoconstriction and vasodilation respectively Noradrenaline in doses of 0 5—3 μg given i v to the recipient likewise caused a reflex flow increase in the isolated cross-circulated calf The peak of this reflex vasodilator response was larger than that which can be seen in the steady state situation after that the sympathetic chain is cut (8—10 ml/100 g tissue and minute Mellander and Johansson 1968) Before adrenergic blockers were given fastigial stimulation caused a muscle vasoconstriction which was similar to that caused by a carotid occlusion eliciting the same blood pressure rise in the recipient (Lisander and Martner 1971) All the mentioned reflex effects in the cross circulated vascular bed as well as the constrictor effects produced by fastigial stimulation disappeared after i a administration of guanethidine (4 mg/kg muscle tissue) or the combination of guanethidine (4 mg/kg tissue) and phentolamine (1 mg/kg tissue) In 3 expts dibenzyline administration (2 mg/kg tissue) gave the same result The corresponding changes in heart rate and blood pressure could still be observed in the unblocked remainder of the recipient In no case did fastigial stimulation alone induce any cholinergic vasodilator fibre activation Even after eserine (0 1 mg/kg estimated tissue weight i a to the crosscirculated limb or the same dose per kg b w i v to the donor) which enhanced the cholinergic

Fig 3 Atropinized cat 3 7 kg paralyzed with gallamine. Intestines are extirpated the adrenomedullary secretion eliminated Both vagi and right sinus nerve are cut while pressure is kept constant in the left carotid sinus throughout Heart rate muscle blood flow resistance in the calf and arterial blood pressure in the *brachial artery are shown in the* figure The arterial perfusion pressure to the calf muscle was kept constant during all stimulations by an adjustable clamp around the abdominal aorta The heavy dots at the bottom of each shaded area comprise the frequency—response curve for defence area stimulation alone (at 1 ms and 4 V) The corresponding upper dots illustrate the situation when a standardized fastigial pressor response (20 Hz 1 ms 0 2 mA) is superimposed Note that the weak neurogenic muscle vasoconstriction induced by defence area stimulation at higher frequencies is more marked when performed together with a fastigial stimulation



dilation caused by defence area stimulation (Lindgren 1955) fastigial stimulation did not produce any dilation in the adrenergically blocked muscle vascular bed

Fig 2 is taken from a cross circulation experiment where guanethidine has been given i v to the donor Defence area stimulation alone results in a marked vasodilation that could be completely blocked by atropine (1 mg/kg b w i v to the donor) The change in recipient arterial pressure during stimulation is negligible Fastigial stimulation alone has no effect on the cross circulated calf muscle vessels where the constrictor fibres are blocked but causes a blood pressure rise in the recipient Heart rate falls somewhat during this stimulation but this was not the case in most experiments Concomitant stimulations of the defence area and the fastigial nucleus result in a muscle vasodilation that is clearly reduced in comparison with that induced when only the defence area is stimulated At the same time the pressure increase in the recipient is somewhat higher than when the fastigial pressor area is stimulated alone In some animals the cholinergic vasodilation could be reduced by 50 per cent in such experiments when the fastigial nucleus was stimulated at 50 and 30 Hz this reduction being less pronounced at lower frequencies It was greatly influenced by dorsoventral changes in cerebellar electrode position as small as 0 5 mm No correlation could be detected between the extent of this fastigial inhibition of the cholinergic vasodilation and the intensity of the pressor response in the recipient Some electrode positions causing vasodilator inhibition were located 0—2 mm dorsally to the fastigial nucleus

In atropinized cats defence area stimulation resulted in a moderate muscle vasodilation The muscle vasoconstriction from fastigial stimulation was not augmented by defence area stimulation suggesting that the latter stimulation might have no influence on the vasoconstrictor fibre outflow to the skeletal muscle vessels To elucidate this further the vagi were cut one carotid sinus was perfused at a constant pressure and the contralateral sinus nerve was crushed in five cats Adrenomedullary secretion was eliminated and the arterial pressure in the hindquarters was kept constant by means of a screw clamp around the abdominal aorta, to avoid myogenic alterations of vascular tone in the calf upon pressure changes In these experiments, where baroceptor activity was kept constant hypothalamic stimulations were performed only posterior to H C coordinates A 12 5 in order to avoid concomitant stimulation of the sympatho inhibitory area (Folkow Johansson and Öberg 1959) Fig 3 is from an experiment in an atropinized animal where the fastigial pressor area was stimulated alone or together with the defence area Note that a weak muscle vasoconstriction occurs when the defence area is stimulated alone at higher frequencies and that simultaneous fastigial activation facilitates this neurogenic constriction There is a powerful mutual facilitation with respect to the blood pressure rise as well —In 5 other expts with an analogous preparation the same type of fastigial facilitation but even more marked was found also in the intestinal and cutaneous vascular beds

Discussion

The interest was focused upon circulatory changes in skeletal muscle in connection with fastigial or hypothalamic defence area stimulations, when performed alone or in combination Stimulation within the rostral pole of the fastigial nuclei regularly induced a blood pressure rise paralleled by a muscle vasoconstriction and an increase in heart rate confirming the results of Miura and Reis (1970) and Achari and Downman (1970) No peripheral vascular effects occur after administration of adrenergic blocking agents indicating that the cholinergic dilator fibres to the muscles are not activated from this cerebellar nucleus

Defence area stimulation alone resulted in the wellknown cholinergic vasodilation in skeletal muscle The vasoconstriction in this tissue induced by fastigial stimulation was similar in magnitude regardless of whether a defence reaction was superimposed or not This is probably due to the fact that in the cat virtually all vasoconstrictor fibre tone must be abolished before substantial sympathetic vasodilator fibre effects can be traced in the skeletal muscles (Folkow Öberg and Rubinstein 1964) Thus under these conditions the fastigial pressor response suppressed the cholinergic dilation but this interference may well be explained by the peripheral organization of the neuroeffector system (see Lisander 1971) and does not necessarily indicate any central nervous modulation of cholinergic dilator fibre activity

However in some experiments neurogenic muscle vasodilation induced by defence area stimulation was almost completely abolished by simultaneous fastigial stimulations that when carried out alone did not produce any significative neurogenic

muscle vasoconstriction One possible explanation to this is that the defence area stimulation facilitated the fastigial action on the vasoconstrictor fibre outflow to the skeletal muscles to such an extent that it now resulted in a hampering of the cholinergic dilation This possibility will be discussed later

In some experiments after the adrenergic blocking drug guanethidine fastigial stimulation sometimes appeared to facilitate the muscle vasodilation induced by defence area stimulation This facilitatory effect was however considerably delayed and is in all likelyhood a result of adrenaline release from the adrenal medulla which is known to occur during defence area stimulation (Grant *et al* 1958) The adrenal release of catecholamines is not easily blocked by guanethidine (Athos *et al* 1962)

This assumption is corroborated not only by the long latency of the peak dilator response but also by the fact that this peak was not reduced by atropine but markedly decreased by β adrenergic blockade Hence although the fastigial pressor response is primarily due to direct action of adrenergic nerves (Achari and Downman 1970) it seems to involve a release of adrenal catecholamines In some situations these amines may considerably modify the neurogenic vascular responses

To clarify whether fastigial stimulation had any influence on vasodilator fibre activity *per se* this activity was studied in a cross circulated and adrenergically blocked calf Adrenergic responses could develop in the unblocked remainder of the recipient In these experiments even intense carotid baroceptor activation did not cause any vasodilation in the cross-circulated calf treated with adrenergic blockers suggesting that the cholinergic vasodiator fibres are not engaged in such reflexes Defence area stimulation on the other hand regularly caused a vasodilation that could be blocked by atropine whereas fastigial stimulation alone had no influence on the calf vessels even after administration of eserine to the cross circulated calf These results therefore indicate that fastigial stimulation alone does not cause any cholinergic dilation

However in these experiments it was found that fastigial stimulation performed simultaneously with a defence area stimulation could partly suppress the vasodilator fibre activation (Fig 2) an effect which in this situation must take place within the central nervous system or possibly at the ganglionic level

Thus the hypothesis that the fastigial pressor area may exert a generally facilitatory influence on the hypothalamic defence reaction was not verified Although the inhibition of reflex bradycardia produced by fastigial stimulation is similar to that which is induced by defence area stimulation the fastigial pressor response appears to be more related to the overall constrictor — response pattern seen when the arterial baroceptors are unloaded (Lisander and Martner 1971)

Sham rage can be elicited by fastigial stimulation in decorticate cats (Zanchetti and Zoccolini 1954) and there is suggestive evidence that the defence area is involved in this type of re ponse (Abrahams Hilton and Zbrozyna 1960) However many procedures known to evoke overall vasoconstrictor—pressor responses have been shown to facilitate sham rage These include chemoreceptor stimulation baro

ceptor unloading and somatic afferent stimulation (see Lisander 1970) It is possible that they all are effective *via* an unspecific activation of the ascending reticular formation

It thus seems clear that fastigial stimulation can suppress the cholinergic vasodilation in skeletal muscle as elicited by defence area stimulation in two principally different ways First whenever the fastigial stimulation excites the constrictor fibre supply to the muscle vessels the impact of these fibres can markedly interfere with the cholinergic dilation at the effector level Secondly the cross circulation experiments strongly suggest that the fastigial stimulation can also at some level within the central nervous system interfere with the activation of the vasodilator fibres The precise mechanism and site of action of this latter interference remains unclear however a similar type of central interference may be elicited from the vermis of the anterior cerebellar lobe (Lisander and Martner 1971)

As to the adrenergic influences proper defence area stimulations did not augment the vasoconstriction in skeletal muscle induced by fastigial stimulation in atropinized cats However a defence reaction may be assumed to activate the arterial baroceptors which would tend to reflexly suppress whatever constrictor fibre tone is present in the skeletal muscle vessels (Djojosugito *et al* 1970) Therefore other animals were prepared so that the baroceptors could be kept at a constant level of activity throughout and the adrenal medullary secretion and autoregulatory counter regulation of the muscle vessels were also eliminated At the same time the cholinergic dilator fibres were blocked by atropine Under these experimental conditions defence area stimulation when performed at high frequencies induced a very weak muscle vasoconstriction and the fastigially induced vasoconstriction could be moderately facilitated by defence area stimulations which could not significantly increase muscle flow resistance Thus it appears that the hypothalamically elicited defence reaction may involve weak activation of vasoconstrictor fibres to the skeletal muscle resistance vessels which is normally suppressed by the secondary baroceptor reflexes Therefore when the defence area alone is activated in the unatropinized animal this increased baroceptor activation secondary to the augmentations in pulse pressure and mean arterial pressure seems sufficient to conceal any element of vasoconstrictor fibre activation completely thereby allowing a cholinergic vasodilation (Lisander 1971) The present experiments indicate however that there is no central suppression of vasoconstrictor fibre tone to the skeletal muscle vessels during defence area activation apart from that reflexly accomplished *via* the baroceptors

We are indebted to A B CIBA Stockholm A B Hassle Goteborg and A B Vitrum Stockholm for generous supply of Ismelin® Aptin® and heparin respectively

References

Abrahams V C S M Hilton and A Zbrozyna Active muscle vasodilatation produced by stimulation of the brain stem its significance in the defence reaction *J Physiol* (Lond) 1960 *154* 491—513

Achari N K and C B B Downman Autonomic effector responses to stimulation of nucleus fastigius *J Physiol* (Lond) 1970 *210* 637—650

ATHOS W J B P McHUGH S E FINEBERG and J G HILTON The effect of guanethidine on the adrenal medulla. *J Pharmacol exp Ther* 1962 *137* 229—234

DJOJOSUGITO A M B FOLKOW P H KYLSTRA B LISANDER and R S TUTTLE Differentiated interaction between the hypothalamic defence reaction and baroreceptor reflexes I Effects on heart rate and regional flow resistance *Acta physiol scand* 1970 *78* 376—385

ECCLES J C M ITO and J SZENTAGOTHAI *The cerebellum as a neuronal machine* Springer Verlag Berlin Heidelberg New York 1967

FOLKOW B B JOHANSSON and B ÖBERG A hypothalamic structure with a marked inhibitory effect on tonic sympathetic activity *Acta physiol scand* 1959 *47* 262—270

FOLKOW B B ÖBERG and E H RUBINSTEIN A proposed differentiated neuro-effector organization in muscle resistance vessels. *Angiologica* 1964 *1* 197—208

GRANT R P LINDGREN A ROSEN and B UVNAS The release of cathechols from the adrenal medulla on activation of the sympathetic vasodilator nerves to the skeletal muscle in the cat by hypothalamic stimulation *Acta physiol scand* 1958 *43* 135—144

KJELLMER I The effect of exercise on the vascular bed of skeletal muscle *Acta physiol scand* 1964 *62* 18—30

LINDGREN P The mesencephalon and the vasomotor system. An experimental study of the central control of the peripheral blood flow in cat *Acta physiol scand* 1955 *35* Suppl 121

LISANDER B Factors influencing the autonomic component of the defence reaction *Acta physiol scand* 1971 *80* Suppl 351

LISANDER B and J MARTNER Cerebellar suppression of the autonomic components of the defence reaction *Acta physiol scand* 1971 *81* 84—95

LISANDER B and J MARTNER Interaction between the fastigial pressor response and the baroreceptor reflex *Acta physiol scand* 1971 *83* 505—514

MELLANDER S and B JOHANSSON Control of resistance exchange and capacitance function in the peripheral circulation *Pharmacol Rev* 1968 *20* 117—196

MIURA M and D J REIS A blood pressure response from fastigial nucleus and its relay pathway in brainstem *Amer J Physiol* 1970 *219* 1330—1336

ZANCHETTI A and A ZOCCOLINI Autonomic hypothalamic outbursts elicited by cerebellar stimulation *J Neurophysiol* 1954 *17* 475—483

Acta physiol scand 1973 87 368—376

From the Department of Physiology Gymnastik och idrottshogskolan Stockholm Sweden

Oxygen Uptake, Muscle High-Energy Phosphates, and Lactate in Exercise under Acute Hypoxic Conditions in Man

By

Howard G Knuttgen and Bengt Saltin

Received 12 June 1972

Abstract

Knuttgen H G and B Saltin *Oxygen uptake muscle high energy phosphates and lactate in exercise under acute hypoxic conditions in man* Acta physiol scand 1973 87 368—376

The relationships among muscle metabolite concentration exercise intensity and oxygen utilization were studied under simulated altitude conditions (decompression chamber 462 mm Hg b p) 5 male subjects exercised in 4 min bouts on a cycle ergometer at a wide range of exercise intensities (range 20—114 % altitude max $\dot{V}_{O_2}$) Oxygen uptake was determined throughout exercise and recovery Muscle biopsies (*vastus lateralis*) and fingertip blood samples were taken at rest and during recovery Muscle ATP and creatine phosphate concentrations became reduced during exercise at all intensities Little or no increase in lactate was observed below approx 60 % altitude max $\dot{V}_{O_2}$ The hypoxic condition caused a decreased acceleration from resting $\dot{V}_{O_2}$ and an increase in O_2-deficit at each absolute intensity as compared with normoxia Close relationships were observed between level of high energy phosphate depletion and both O_2-deficit and fast component of O_2-debt not different from the same relationships under normoxic conditions Virtually all differences in physiological response to identical exercise intensities at altitude as compared to normoxia were appropriately adjusted by expressing exercise intensity relative to the respective aerobic power capacities in the two conditions Physiological responses to this type of exercise appear determined in great part by the availability of oxygen

The relationship of skeletal muscle metabolism to exercise intensity has been previously investigated under normoxic conditions (Hultman Bergstrom and McLennan Andersson 1967 Karlsson Diamant and Saltin 1971 Knuttgen and Saltin 1972) Both ATP and creatine phosphate (CP) concentrations became reduced throughout a range of mild to exhaustive exercise with greater reductions at the higher intensities At approximately 60 % of maximal aerobic power it was observed that as the intensity increased lactate began to appear in increasingly larger concentrations in both active muscle and in the blood In this same range both the oxygen deficit and the oxygen debt demonstrated marked increases

Ascent to altitude which causes a decrease in arterial oxygen tension and content could be expected to have definite effects on muscle metabolism A decrease in a person s maximal aerobic power as a result of the lowered partial pressure of atmos-

TABLE I Personal data and Max $\dot{V}_{O_2}$ for the subjects

Subj	Age	Ht cm	Wt kg	760 mmHg max $\dot{V}_{O_2}$ $l \times min^{-1}$	462 mmHg max $\dot{V}_{O_2}$ $l \times min^{-1}$
NC	26	187	82	4 36	3 30
ED	24	181	80	4 05	3 50
BD	23	184	79	3 80	3 20
LE	20	195	83	4 70	3 57
PS	23	180	74	3 70	3 00

pheric oxygen results in any absolute exercise intensity and oxygen uptake becoming of greater magnitude relative to the new maximal oxygen uptake

The purpose of the present study was to investigate the effects of acute exposure to hypoxia (simulated altitude 462 mm Hg b p or 4000 m) upon the relationships among exercise intensity oxygen utilization and muscle metabolism.

Subjects and Methods

5 healthy male subjects all in an above average state of physical condition and endurance training were employed All subjects underwent pre testing for determination of maximal oxygen uptake (max $\dot{V}_{O_2}$) during normoxic condition and during acute exposure to ambient hypoxia (simulated altitude in a decompress on chamber) Personal data of the subjects and *max* $\dot{V}_{O_2}$ *under the two* conditions are presented in Table I Each subject then took part in 3 expts at simulated altitude 3 subjects (NC BD LE) worked at 15 45 and 75 of sea level maximal aerobic power and 2 subjects (ED PS) at 30 60 and 90 The relative exercise intensities are expressed in the following discussion in terms of the per cent of the respective maximal oxygen uptakes each intensity demanded under steady state conditions (submaximal exercise) or the assumed $\dot{V}_{O_2}$ at a mechanical efficiency of 23 (supermaximal exercise)

Exercise consisted of cycling for exactly 4 min at 50 rpm on a cycle ergometer (von Dobeln 1954) which had an armchair attached so that the subject sat comfortably behind and at the same level as the pedal axle This exercise position was employed *to facilitate continuous measurements of* recovery following exercise The mechanical *efficiency for cycling exercise* during steady state in this position averaged 23 and was not different from that observed for cycling in the conventional manner

After simulated altitude had been attained in the chamber each experiment began with a 30-min rest period during which time a 20–30 mg muscle sample was taken from the lateral portion of the quadriceps by needle biopsy (Bergstrom 1962) and a blood sample taken from a hyperemic finger tip The subject then assumed the sitting position in the cycle armchair When resting heart rate had again been attained a 5 min collection of expired air (Douglas bag method) was made The subject then performed 4 min of exerc*ise with expired air continuously collected (0–3 3–4 min)* This was followed by 45 min of reco*very with the subject still in the sitting pos*ition and with expired air collection throughout

Muscle biopsies and blood samples were also taken immediately after and 6 min after cessation of pedaling An additional blood sample was obtained 45 min after exercise Muscle biopsies were frozen within 5–7 s in liquid nitrogen (post exercise biopsies 5–7 after termination of exercise) and later analyzed according to methods described by Lowry and co-workers (1964) and modified by Karlsson (1971) for the following metabolites ATP CP glycogen and lactate Blood lactate concentration was determined by a micro-modification of an enzymatic method (Scholz *et al* 1959) Oxygen uptake was calculated *following analysis of expired ai*r by a modified Haldane technique Oxygen deficit was calculated as the *difference between the total oxygen taken* up during the 4 min exercise period and the normal steady state value for each subject at the particular exercise intensity

Oxygen debt was calculated as the oxygen taken up in recovery in excess of resting values in the sitting position The fast component of oxygen debt was calculated as the oxygen taken up during the

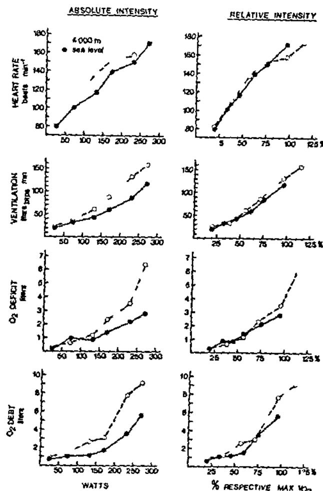


Fig. 1. Mean values at 4000 m (present study) and at sea level (data from Knuttgen and Saltin 1972) for steady state heart rate, steady state pulmonary ventilation, oxygen deficit and oxygen debt in relation to absolute (left column) and relative (right column) exercise intensity. Relative intensities are calculated as the elicited steady state V_{O_2} in percent of altitude and sea level max V_O respectively. The highest relative intensity at altitude is presented as percent of altitude max V_O represented by the V_O demand at this intensity assuming 23% mechanical efficiency.

first 6 min of recovery in excess of an extrapolation of a semilogarithmic plot of $\dot V_O$ from 6–45 min of recovery.

The reproducibility of the methods used in the present study was as follows: submaximal oxygen uptake ±1.5%, maximal oxygen uptake ±3%, blood lactate ±3, ATP ±8, CP ±5, glycogen ±6 and muscle lactate ±5 (SE in percent of Mean of duplicate analyses).

Identical experiments to those of the present study had been performed 2–3 weeks previous under normoxic conditions (Knuttgen and Saltin 1972). The latter study included the 5 subjects employed in the present study and in certain of the figures which follow the normoxic data of these 5 subjects have been included for comparison.

Results

The average decrease in maximal aerobic power (max V_{O_2}) for the subjects at altitude was 19% (range 13–24%) (see Table I). When absolute exercise intensity was expressed as opposed to normoxia the relative stress (% of max) was therefore higher in each case at altitude. O_2 uptake after approximately 6 min exercise evidences no difference for the same absolute intensities in hypoxia. This was confirmed with the

TABLE II Individual values for exercise intensity oxygen uptake (4th min of exercise) and metabolite concentrations for the 15 expts under hypoxic conditions Values at rest represent mean values for 3 expts Values for metabolites in the exercise conditions represent immediate post exercise for each experiment When changes from rest are presented in Fig 3 4 and 6 the resting values of the individual experiments were employed in the calculations

Subject	Exerc Intens W	$\dot{V}_{O_2}$ l × min	ATP mmoles × kg^{-1}	CP mmoles × kg^{-1}	Musl Lactate mmoles × kg^{-1}	Bld Lactate mmoles × l^{-1}
NC	Rest	0 34	4 8	19 7	1 1	1 7
	26	0 67	4 8	18 3	2 9	1 1
	130	1 95	4 5	10 7	6 1	3 5
	230	3 04	1 9	3 6	18 3	4 6
BD	Rest	0 36	4 8	14 8	1 2	1 4
	25	0 85	4 3	14 6	1 4	1 8
	115	1 69	5 2	12 1	2 7	2 2
	215	2 65	3 5	3 2	20 0	8 5
LE	Rest	0 37	5 4	19 8	0 8	1 5
	25	0 73	4 2	13 4	0 9	0 8
	145	2 11	3 7	12 2	1 3	2 3
	245	3 00	2 9	3 9	20 2	10 9
ED	Rest	0 35	4 6	17 2	0 4	0 9
	80	1 35	4 7	16 5	1 4	1 4
	180	2 61	2 7	7 7	6 2	3 7
	280	3 02	2 0	4 5	28 6	17 1
PS	Rest	0 42	4 5	18 0	1 3	1 1
	90	1 10	3 7	12 0	1 1	1 0
	180	2 11	3 0	8 8	7 8	5 9
	295	2 66	2 5	2 6	16 7	12 0

subjects in the present study during pre testing in 10 min bouts of exercise The acceleration of O_2 uptake to steady state in exercise was retarded in hypoxia as evidenced by consistently lower values for both the initial 3 min and also the 4th min of exercise In normoxia steady state had been attained by the end of the 3rd min of exercise

A comparison of various parameters hypoxia and normoxia (altitude and sea level) is presented in Fig 1 *vs* absolute and relative exercise intensity When mean values for heart rate ventilation O_2 deficit and O_2 debt were compared *vs* absolute exercise intensity (left column) a discrepancy occurred in which the various responses at altitude consistently exceeded normoxic responses especially at the higher intensities The values *were quite consistent with* each other when plotted against percent of the maximal aerobic power appropriate to the respective condition

Certain of the biochemical results from the individual experiments are presented in Table II There were reductions in concentration of both ATP and CP at all levels of exercise the reductions being generally proportional to exercise intensity

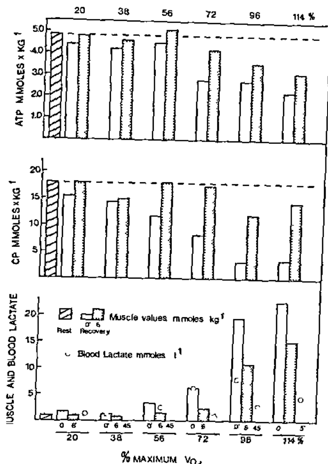


Fig 2 Average concentrations of muscle ATP (upper panel) muscle CP (middle panel) and muscle and blood lactate (lower panel) at rest immediate post exercise and after 6 min recovery Values for blood lactate are also presented for 45 min recovery Values at rest (diagonally marked bars and in upper panels broken lines) represent averages for all 15 expts

in both cases A complete picture of the exercise and recovery pattern in muscle metabolites is presented in Fig 2 At the higher exercise intensities where greater concentration reductions were observed for ATP and CP return to resting values did not occur during the first 6 min of recovery

At the same absolute work load most individual data on the change from resting concentration in high energy phosphate demonstrated a more marked depletion at altitude as compared to sea level (Fig 3) However this difference was diminished when the exercise was expressed in relative terms as also illustrated in Fig 3 The relationship of the change in high energy phosphate to deficit appeared consistent through the range of deficits of comparable magnitude (Fig 4) Further reduction in phosphagen concentration failed to occur at a depletion of approximately 16 mmol × kg^{-1} in spite of a larger deficit

Muscle lactate concentration showed no increase at the two lower exercise loads but at a relative load demanding 56 % of max V_{O_2} a significant increase was noticed. At the two heaviest exercise intensities demanding just below and just above max

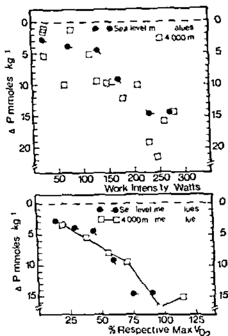


Fig 3

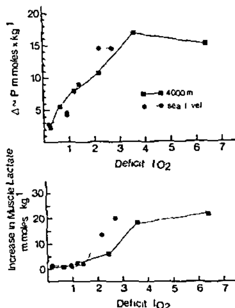


Fig 4

Fig 3 Change n h gh energy pho ph te (ATP and CP) fron rest to immediate post exerc e n relation to absolute (upper pan l) and relative (lower panel) work intensity Ch nges n h gh energy pho ph te w re calculated from resting alues for each exper ment and are exp essed n mmol s per kg wet muscle

Fig 4 Relat onsh p of total high energy pho phate conc nt ation change (upper p nel) and muscle lactate concentrat on ch nge (lower panel) in exe cise to the oxygen deficit observed during the t ans t on f om rest to steady state V_{O_2} Mean values for 4000 m experiments are p esented along with mean values for sea level exper ments (Knuttgen and Saltin 1972) conducted at identical ab olute xe c nten t es

V_{O_2} muscle lactate concentrations of 19 and 24 mmol × kg⁻¹ × min⁻¹ respectively were reached (Fig 2) In these instances a marked gradient was observed between muscle and blood lactate concentration

6 min recovery did not bring muscle lactate concentrations at the 3 heavier intensities back to resting level The discrepancies between muscle and blood were diminished during this period with concentrations attaining near equilibrium values At the highest intensities blood lactate was still elevated after 45 min recovery but had attained resting levels for the intermediate intensities at this time Above a deficit of approximately 1 5 l muscle lactate increases were observed to be directly proportional to intensity but of lesser magnitude in the altitude experiments (Fig 4)

Comparing the complete picture for muscle and blood lactate concentrations during exercise at sea level and altitude conditions revealed that only at the two

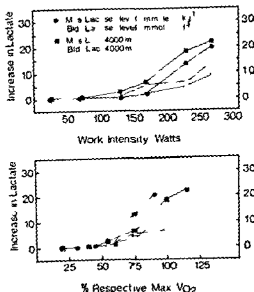


Fig 5

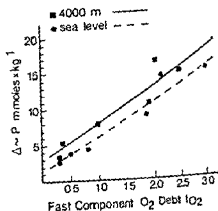


Fig 6

Fig 5 Change in muscle and blood lactate concentrations at 4000 m and at sea level (Knuttgen and Saltin 1972) in relation to absolute (upper panel) and relative (lower panel) exercise intensity Mean values are presented

Fig 6 Relationship of change in high energy phosphate concentrations (from rest to immediate post exercise) to calculated fast component of oxygen debt Mean values for experiments performed at identical absolute exercise intensities and lines of regression are presented for 4000 m and for sea level experiments (Knuttgen and Saltin 1972)

heaviest intensities were higher muscle and blood lactate values attained at altitude (Fig 5) At the same relative intensities muscle lactate was lower at altitude as compared to sea level at an O_2 uptake above 60 % The blood lactate concentration however was not significantly different at the exercise intensities studied

It has been hypothesized that the return to resting levels by the high energy phosphates in muscle is one of the processes occurring during the first few minutes of recovery which is related to the fast component of the oxygen debt Strong positive relationships existed in both the hypoxic and normoxic data between the two parameters (Fig 6)

Discussion

It has been shown that under normoxic conditions a strong positive relationship exists between exercise intensity and the acceleration of V_{O_2} during the transition from rest to exercise both at submaximal intensities (Knuttgen 1971) and at intensities exceeding max V_{O_2} (Åstrand and Saltin 1961) In the present study the ambient hypoxic condition resulted in an opposite reaction

The same absolute work load in hypoxia which had become a greater relative stress resulted in a slower acceleration in V_{O_2} and greater O_2 deficit This observation has not been reported previously

The relationship of O_2 deficit to muscle ATP and CP depletion and to muscle lactate accumulation in normoxia reported by Karlsson (1971) and later comfirmed (Knuttgen and Saltin 1972) held true for the hypoxic condition The larger deficits observed at the same absolute intensities corresponded directly to greater phosphagen depletion in muscle and in the higher intensities where lactate changes are observed, to greater accumulation of lactate in muscle

While the turnover of ATP and CP seems to take place at concentration levels proportional to exercise intensity the return to resting levels is apparently a slower process than previously imagined both at sea level (Knuttgen and Saltin 1972) and at altitude A portion of the O_2 debt fast component might very well correspond to oxygen spared by energy gained in the breakdown and reduced concentrations of both ATP and CP during exercise The involvement of the two compounds in the processes of recovery does not result in a rapid return to resting values however

Maximal aerobic power was lowered as a result of the simulated altitude condition an average of 19 % an observation consistent with previous reports (*e g* Johnson 1967) The effect which lowered arterial O_2 tension and content has on the availa bility of O_2 in muscle tissue at various submaximal exercise intensities appears proportional and directly related to the accompanying lowering of maximal aerobic power The observation that expressing exercise intensity in relative terms virtually eliminated discrepancies in metabolite concentration and certain O_2 utilization changes comparing hypoxic with normoxic responses may indicate a close relationship of these responses to the availability of oxygen When physiological regulation during acute exposure to hypoxia is considered attention must be paid to the observation that changes in response become strikingly predictable when the variables are related to the relative stress

Despite the enhanced responses of the various parameters in the hypoxic condition the relationships among the variables remained quite consistent The increases in O_2 deficit were accompanied by appropriate increases in O_2 debt The increased *deficits were also accompanied by the appropriate increases in phosphagen depletion* and at the highest intensities increased lactate production The increased phosphagen depletions were in turn accompanied by appropriate increases in the fast component of O_2 debt

Lactate also appeared in muscle and blood at similar relative intensity (approxi mately 60 % of respective max V_{O_2} and corresponding O deficit of 1 5 l) While blood values were not appreciably different at similar relative intensities above this level muscle values were not consistent At similar absolute exercise intensities there was a greater accumulation while at similar relative intensities (and deficits) there was a smaller accumulation of muscle lactate in hypoxia The consistency of blood lactate values at similar relative intensities has been observed previously (Hermansen and Saltin 1967) The differences observed in muscle values in the present study would indicate that as the total energy production is lower at the same relative stress in hypoxia the proportional contribution of anaerobic glycolysis is similar

When compared on the basis of relative intensity where deficits became equal

muscle lactate concentrations then became proportionately lower in hypoxia It is still conceivable that glycolysis was quantitatively the same under the two conditions The hypoxic condition could result in pyruvate being oxidized or metabolized to alanine or malate instead of being reduced to lactate Possibly a more likely explanation is that identical rates of glycolysis result in identical lactate production but the greater cardiac output in the acute hypoxic condition (Stenberg Messin and Ekblom 1967) results in a faster removal of the lactate The similarity of the blood lactate concentrations at the same relative intensity observed in this study is consistent with this explanation There are no data available on the turnover of the blood lactate in the acute hypoxic condition but the present data do not negate the possibilities of an unchanged or even reduced uptake from the blood by other tissues and organs

H G Knuttgen was on leave of absence from the College of Allied Health Professions and Dept of Biology Boston University and was the recipient of a research grant from the Boston University Graduate School This study was supported in part by a grant from the Délégation Generale à la Recherche Scientifique et Technique France and the Swedish Medical Research Council Project 40X 2203

References

ÅSTRAND P O and B SALTIN Oxygen uptake during the first minutes of heavy muscular work *J appl Physiol* 1961 *16* 971–976
BERGSTRÖM J Muscle electrolytes in man *Scand J clin Lab Invest* 1962 *Suppl 68* 110
VON DÖBELN W A simple bicycle ergometer *J appl Physiol* 1954 *7* 222–224
HERMANSEN L and B SALTIN Blood lactate concentration during exercise at acute exposure to altitude in *Exerci at Altitude* (ed R Margaria) *Excerpta Medica* 1966 48–53
HULTMAN E J BERGSTROM and N MC LENNAN ANDERSSON Breakdown and resynthesis of phosphorylcreatine and adenosine triphosphate in connection with muscular work in man *Sand J clin Lab Invest* 1967 *19* 56–66
JOHNSON Jr R L Pulmonary diffusion as a limiting factor in exercise stress *Circulat Res* 1967 *0* –160
J Lactate and phosphagen concentrations in working muscl f n an *1 ta phys l and* 1971 Suppl 358
KARLSSON J B DIAMANT and B SALTIN Muscle metabolites during submaximal and maximal exercise in man *Sand J lin Lab I vest* 1971 *26* 385–394
KNUTTGEN H G Oxygen debt after submaximal physical exercise *J appl Physiol* 1970 29 651–657
KNUTTGEN H G and B SALTIN Muscle metabolites and oxygen uptake in short term submaximal exercise in man *J appl Physiol* 1972 *32* 690 694
LOWRY O H J V PASSONNEAU F X HASSELBERGER and D W SCHULZ Eff t of ischemia on known substrates and cofa t rs f the glyc lytic pathway in brain *J biol Chem* 1964 *239* 18–30
SCHOLZ R H SCHMITZ T BUECHER and T O LAMPEN Üb r die Wi kung n Nystatin auf Bockerhefe *Biochem Z* 1959 *331* 71 86
STENBERG J R MESSIN and B EKBLOM Hemodynamic response to w rk at mulated altitude 4 000 m *J appl Physiol* 1966 *21* 1155 1166

Acta physiol scand 1973 87 377—390

From the Department of Physiology University of Wisconsin Madison Wisconsin 53706 USA

An Investigation of the Action of Ouabain on the Sodium Efflux in Barnacle Muscle Fibres

By

E EDWARD BITTAR STEPHEN S CHEN BO G DANIELSON and EDMUND Y TONG

Received 18 June 1972

Abstract

BITTAR E E S S CHEN B G DANIELSON* and E Y TONG *An investigation of the action of ouabain on the sodium efflux in barnacle muscle fibres* Acta physiol scand 1973 *87* 377—390

Measurements of radiosodium efflux were carried out on single fibres from the barnacle *Balanus nubilus* or *B aquila* using the micro-injection technique 10^{-4} M ouabain caused an average fall of 68 % of the Na efflux the minimal effective concentration being *ca.* 10^{-7} M Internal application of 4×10^{-3} M ouabain was without effect Abolition of the ouabain sensitive component with ouabain, followed by raising the external K concentration to 30 mM resulted in a marked rise in the residual Na efflux Stimulation of the ouabain insensitive Na efflux was also seen after reducing the external pH as well as after microinjecting $CaCl_2$ but not $MgCl_2$ *Uncoupling of the active transport system by K removal followed by external* application of ouabain and subsequent acidification of the bathing medium, led to stimulation which was considerably greater than that taking place when the Na efflux was uncoupled by K removal and then stimulated by external acidification.

This paper is concerned with the chemical nature of the mechanism pumping sodium out of the barnacle muscle fibre and with the question whether the behaviour of the Na efflux into a K free solution is affected by ouabain Working with red cells Garrahan and Glynn (1967) showed that ouabain is additionally able to reduce the Na efflux into a K free solution by about one half This finding provided some justification for the idea that the ouabain sensitive transport system is involved not only in the catalysis of Na K exchange but also in the one to one exchange the so called Ussing type of exchange diffusion However such an idea cannot be reconciled with the conclusion arrived at by Hoffman (1966) that the Na pump of the red blood cell is divided into 2 quite independent pumps both of which are metabolically driven In the absence of any precise information about the nature of the Na efflux mechanism in single red blood cells it is obviously important to study

* Permanent address Institute of Physiology and Medical Biophysics Uppsala University Uppsala Sweden

such a complex problem by employing single giant cells e g the barnacle muscle fibre

The present work was carried a stage further to establish some of the basic properties of the ouabain insensitive Na efflux This information was obtained in three different ways First the effect of high K on the ouabain insensitive Na efflux was compared with the effect of high K on the Na efflux from unpoisoned fibres Secondly the effect of low external pH on the ouabain insensitive Na efflux was tested This seemed of interest particularly since earlier studies by Bittar and Tong (1971) revealed that barnacle fibres are extremely sensitive to acidification of the bathing medium And thirdly $CaCl_2$ was micro injected in the hope of settling the question whether the internal free Ca concentration regulates the size of the ouabain insensitive Na efflux

A brief account of part of this work has already appeared (Danielson *et al* 1971 *a b*)

Methods

Materials Specimens of *Balanus nubilus* or *B aquila* were supplied by the Pacific Biomarine Co in Venice California They were maintained in a 150 gallon aquarium containing aerated artificial sea water at about 12 C

Dissection Single fibres were dissected in the usual way from one of the flexor muscle bundles severing the shell end not under but out of the artificial sea water They were then cannulated in the same way as crab muscle fibres (Caldwell and Walster 1963) Observation indicated no signs of deterioration over periods of 1 1/2 to 3 h The initial membrane potentials were retained in addition the fibres contracted when depolarized with high K and remained very sensitive to acidification of the bathing medium

The microinjector used The microinjector was of the pattern constructed by Hodgkin and Keynes (1956) as modified by Caldwell and Walster (1963) The inner capillary of the device was 110 120 *um* in diameter The column of test fluid ejected into these fibres was usually 1 cm in length Assuming that the intrafibre water volume of fibres 30 20 and 15 mm in length and 1 3 mm in diameter is about 25 17 and 12 5 *ul* respectively the dilution factor by volume may then be taken to be 250- 170 and 125 times A number of the control experiments designed to see whether injury by the microinjector alters the behaviour of the Na efflux were done with an inner capillary 130 *um* in diameter Occasionally the procedure of loading the fibre with Na resulted in partial contracture lasting roughly one min These fibres were not discarded However fibres which showed a sustained contracture were discarded

Solutions used The experiments were performed in artificial sea water having the following composition (mM) NaCl 465 KCl 10 $CaCl_2$ 10 $MgCl_2$ 10 $NaHCO_3$ 10 and pH 7 8 K free or high K solutions were prepared by substituting or reducing the NaCl in equimolar amounts The solutions of ouabain used were always fresh Ouabain was obtained from Sigma Chemical Company

Congo red methyl red and ruthenium red dyes dissolved in water were used as markers in an attempt to explore the possibility that injury of these fibres by the microinjector may lead via the T system to the development of channels between the intra and extrafibre fluid phases

Radioactivity measurements NaCl in aqueous solution was supplied by Amersham Searle Co (SKS 1) The solution was dried down and then made up to a small volume so that volumes in the order of 0 1 *ul* gave roughly 50 000 *cpm* This amount of activity proved to be quite adequate for experiments of the type reported here

The procedure used for collecting the efflux samples and counting the activity of the effluent and the activity remaining in the fibre at the end of each experiment was basically as described by Bittar (1966) A well type counter (*Panax model* CsW1) fitted with a solid phosphor connected to a scaler manufactured by Nuclear Chicago was employed

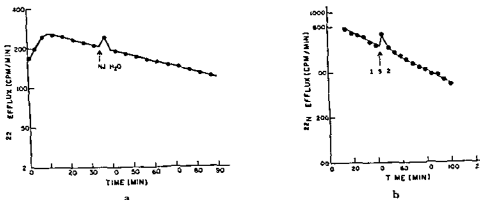


Fig 1 a. Sodium efflux from a barnacle fibre bathed in artificial sea water before and after the microinjection of a 1 cm column of destilled water plotted semilogarithmically The inner capillary used was 110 μm in diameter and penetration into the fibre when loading with Na and subsequently injecting it with distilled water did not exceed 1 cm

Fig 1 b Sodium efflux from a barnacle fibre before and after being injected with distilled water The fibre was loaded by inserting the inner capillary measuring 110 μm in diameter to a depth of approximately 1 cm followed by ejection of a 1 cm column of hot solution The second injection involved penetration of the fibre to a depth of 2 cm and the ejection of a 1 cm column of distilled water

Results

Control experiments The procedure of microinjection was always carried out on fibres temporarily suspended in air and not in the bathing medium The procedure consisted in directing the microinjector down the axis of the cannulated fibre to a depth of at least 1 cm A 1 cm column of test fluid was then discharged and the inner capillary withdrawn by lowering the Perspex platform with the help of the Palmer screw stand The whole manoeuver was usually complete within one min

In the experiment recorded in Fig 1 a the fibre was loaded with ²²Na using a microinjector with an inner capillary 110 μm in diameter Penetration into the fibre was limited to about a 1 cm depth It will be seen that the injected Na fully equilibrated with the free internal sodium in a matter of 10—15 min and that the loss of radiosodium followed simple exponential kinetics 35 min after loading the same microinjector was reinserted to a depth of 1 cm and a 1 cm column of distilled water was discharged As shown in this figure the manoeuver led to no change in the course of the Na efflux (15 expts) Observation based on a large number of experiments however showed frequent occurrence of a small but transitory rise in the Na efflux This was readily accounted for by the time occupied in completing the injection procedure

It is not difficult to envisage that reintroducing the microinjector causes further damage to the fibre This is because the track formed by the initial injection will have already partially collapsed and because the syringe may or may not have occupied the same track The latter possibility is not so remote in view of the

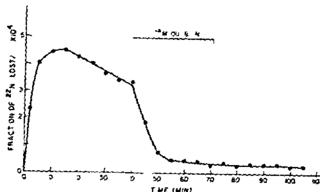


Fig 2 The rate coefficient for Na efflux from a barnacle fibre treated with 10^{-4} M ouabain

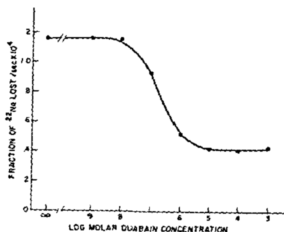


Fig 3 The rate coefficient for Na efflux from a barnacle fibre during treatment with ouabain plotted against the log concentration Since the response to 10^{-3} M ouabain was diphasic the lowest value obtained was plotted

evidence marshalled from experiments with dyes *e g* congo red methyl red and ruthenium red The second injection of fibres with any of these dyes was associated with the appearance of a second streak of colour only partly overlapping the first streak The point of importance of these studies is that in none of them has lateral loss of the dye or loss into the bathing medium been seen

It could be objected that reinsertion of the microinjector to a depth of 1 cm does not bring about enough new damage to the fibre This is then why a series of experiments were done in which the microinjector was reintroduced twice first to a depth of 1 cm and then to a depth of 2 cm None of these 8 expts produced evidence of alteration in the behaviour of the Na efflux This was also true of 5 expts in which the microinjector was reinserted to a depth of 2 cm thereby leading to fresh damage over a 1 cm length of the fibre As illustrated by Fig 1 b the Na efflux was completely unaffected by this manoeuver Additional attempts involving the use of an inner capillary 130 μm in diameter to produce greater damage during the second injection procedure further confirmed that the procedure of microinjection is innocuous insofar as the sodium efflux mechanism is concerned One reason

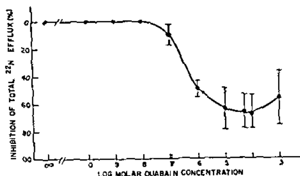


Fig 4 The dose response curve for a large number of fibres obtained from the same barnacle specimen The abscissa has log scale The average inhibition caused by ouabain ±1 S D is plotted The curve is based on the results of the following number of experiments 10^{-10} M 3 10^{-9} M 3 10^{-8} M, 3 10^{-7} M 7 10^{-6} M 6 10^{-5} M 14 5×10^{-5} M 21 10^{-4} M 23 10^{-3} M 6

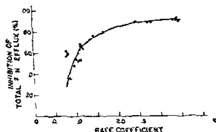


Fig 5

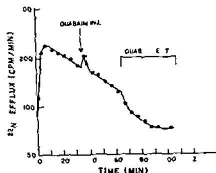


Fig 6

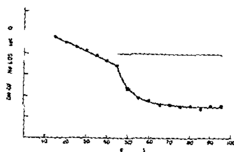
Fig 5 The rate coefficient for Na efflux estimated prior to applying 10^{-4} M ouabain plotted against the percentage inhibition caused by *ouabain* The curve was drawn according to best visual fit

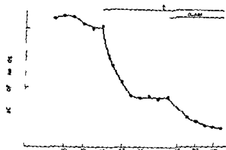
Fig 6 Sodium efflux from a barnacle fibre before and after internal application of 4×10^{-3} M ouabain followed by *external application* of 10^{-4} M ouabain

able explanation of these results is to imagine that the microinjector causes damage mainly to the T system but that the damaged tubules collapse and become plugged with debris and protein coagulum The result is the sealing off of what would otherwise have been an extra leaky fibre membrane

Effect of ouabain on the Na efflux

The effect of 10^{-4} M ouabain is illustrated in Fig 2 As is seen external application of ouabain produced a large fall in the Na efflux but maximal action occurred rather slowly This mode of behaviour towards ouabain resembles that found by Bittar *et al* (1967) in crab muscle fibres These experiments with 10^{-4} M ouabain have also shown that a 30 min exposure to ouabain is enough to bring about an irreversible effect lasting at least 2 h when the solution is replaced by a ouabain free medium It will be remembered that this is also true of squid axons (Caldwell and Keynes 1959)





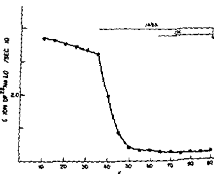


Fig. 7 a. The rate coefficient for Na efflux from a barnacle fibre before and after omitting external K from the bathing medium.

Fig. 7 b. The rate coefficient for Na efflux from a barnacle fibre before and after omitting external K ions followed by external application of 10^{-4} M ouabain.

Fig. 7 c. The rate coefficient for Na efflux from a barnacle fibre treated with 10^{-4} M ouabain followed by K removal.

The next step was to establish both the minimal and maximal effective concentrations of ouabain. Fig. 3 illustrates the inhibition curve for Na loss obtained by treating a fibre with graded concentrations of ouabain. Significantly, inflexion of the curve occurs when a concentration of ouabain greater than 10^{-8} M is introduced into the bathing medium and maximal inhibition is found to develop within 30 min with a concentration of 10^{-4} M or 10^{-3} M. Two interesting points emerge from these results. One is that barnacle fibres are far more sensitive to ouabain than crab muscle fibres. The other is that low concentrations of ouabain produce inhibition rather than stimulation of Na transport. This is not always so with other preparations, e.g. toad bladder (McClane, 1965).

A quantitative treatment of the results indicates that the magnitude of the inhibitory action of ouabain as calculated on the basis of the change in rate constant varies rather widely. This is shown in Fig. 4, which gives the inhibition curve for a very large number of experiments. It can be seen that ouabain in a concentration of 10^{-5} M caused a diphasic effect, viz. a fall in the Na efflux followed 20 min later by a steady rise in the loss of Na*. Estimates of the magnitude of the effect caused by 10^{-4} M ouabain give an average value of 68 %, with a range of 47—93 %. This is not as high as that reported by Brinley (1968).

A special feature of the results obtained with 10^{-4} M ouabain is that fibres exhibiting rate constants which were a constant behaved as if they were more sensitive

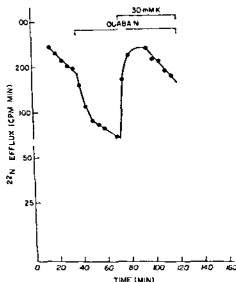


Fig 8 Stimulation of the Na efflux by a 30 mM K solution following treatment of a barnacle fibre with 5×10^{-5} M ouabain

to the inhibitor than fibres exhibiting a gradual decline in the rate constant This is indicated by Fig 5 which is a plot of the percentage inhibition versus the rate coefficient for Na efflux Clearly fibres with high rate constants which continued to be constant were extremely responsive to ouabain (90—95 % inhibition) However in the majority of the fibres tested the magnitude of the effect produced by 10^{-4} M ouabain turned out to be less This held for the fibres in which the rate coefficient for Na efflux always seemed to fall off and in which some fibres showed little sensitivity to ouabain One way of interpreting this result is to suppose that the degree of ouabain sensitivity is related to the amount of free internal Na rather than to the total internal Na That this may well be the case is encouraged by the observation that fibres showing high rate constants which remained a constant failed to reveal the presence of a significant fraction of sequestered or bound Na The bound fraction of Na was calculated according to the slope ratio method as put forward by Dick and Lea (1967)

Lack of effect of internally applied ouabain

Having found that external application of ouabain interrupts the Na efflux it seemed natural to enquire if the transport enzyme behaves in a similar way when ouabain is applied internally A typical result for 5 expts as given in Fig 6 shows quite clearly that the microinjection of 4×10^{-3} M ouabain was without effect on the Na efflux When however 10^{-4} M ouabain was applied externally the Na efflux fell appreciably Identical findings have been reported by Bittar *et al* (1967) for crab muscle fibres and by Caldwell and Keynes (1959) for squid axons

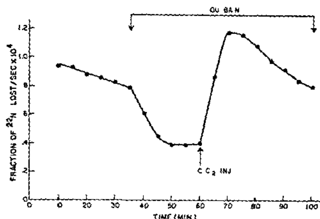


Fig 9 The rate coefficient curve for Na efflux from a barnacle fibre treated with 5×10^{-5} M ouabain followed by internal application of 1 M $CaCl_2$

The effect of K removal

It now seemed necessary to find out whether active Na extrusion depends upon the presence of external K ions An important point is that Edwards and Harris (1957) found the effect of K removal on frog muscle to be comparable to that obtained by strophanthidin The present explanation for this in terms of the transport enzyme though straightforward fails to take into account three possibilities First that in activation of the transport enzyme may result in the emergence of the Ussing mechanism the magnitude of which was well depend *inter alia* on the effectiveness f ouabain Secondly not all K ions may be removed from the immediate surround gs of the muscle fibre by simply replacing the bathing medium with a K free solution Thirdly there may exist in the Na pump a metabolic component as evidenced for example by the large rise in the Na efflux caused by acidifying the external medium (Bittar and Tong 1971) In view of these considerations 2 types of experiments were done The aim of the first type was to test the effect of K removal followed by the external application of ouabain In the second group of experiments ouabain was applied followed by K removal As shown in Fig 7 *a* K removal caused a large reduction in the Na efflux (15 expts) Estimates of the size of the fall in the Na efflux indicate it to be on average 56 % with a range of 48—66 % This agrees with the results of Brinley (1968) for barnacle muscle fibres and Bittar *et al* (1967) for crab muscle fibres Shown in Fig 7 *b* is the effect of K removal followed by 10^{-4} M ouabain (6 expts) In these experiments the inhibitory action of ouabain amounts to 12 % with a range of 3—27 % The simplest explanation of this is that the action of K removal on the active transport system is incomplete and that ouabain abolishes the residual active Na efflux Alternatively ouabain may inactivate a K independent moiety of the transport enzyme That this is unlikely is shown by the second experiment in which ouabain was first applied externally As illustrated by Fig 7 *c* 10^{-4} M ouabain caused a

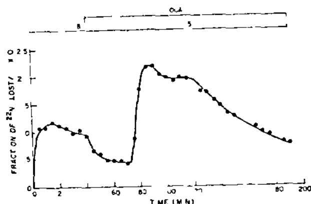


Fig 10 The rate coefficient for Na efflux from a barnacle fibre treated with 5×10^{-5} M ouabain followed by lowering the external pH to 5 8

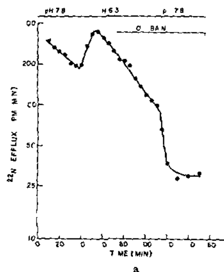
sharp reduction in the emergence of Na while K removal caused hardly any further change in the behaviour of the residual Na efflux (7 expts)

Effect of high K and micro injected CaCl on the ouabain insensitive Na efflux

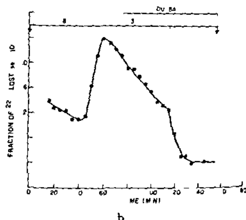
When fibres were poisoned with 5×10^{-5} M ouabain and then the external K concentration raised from 10 to 30 mM the Na efflux was found to greatly rise (6 expts) as Fig 8 shows This was not astonishing in view of two pieces of evidence first that depolarization of the barnacle fibre membrane is associated with an increased Ca influx (Hagiwara and Naka 1964) and second that micro injection of CaCl into barnacle fibre results in a large rise in the Na efflux (Bittar *et al* 1971) The possibility of there being a close relationship between the size of the ouabain insensitive Na efflux and the internal free calcium concentration was there fore investigated (5 expts) A typical experiment is given in Fig 9 This clearly shows that when 1 M $CaCl_2$ was micro injected shortly after the fall in the Na efflux caused by 5×10^{-5} M ouabain there took place a huge rise in the loss of Na Since injections of MgCl (5 expts) were ineffective it seems safe to infer that the activat ing species is Ca and not Cl

The response of the Na efflux to acidification

As reported by Bittar and Tong (1971) a notable feature of the Na efflux in barnacle fibres is its marked sensitivity to a low external pH Since stimulation of the Na efflux by acidification also occurs in the absence of external K ions the possibility arises that protons or CO influence the ouabain insensitive portion of the Na pump In order to check the validity of this inference 10 expts were carried out *in which the bathing medium was acidified after* poisoning the fiber with ouabain The result given in Fig 10 shows that lowering the external pH to 5 8 caused a great rise in the residual Na efflux following inactivation of the ouabain sensitive compo nent with 5×10^{-5} M ouabain However one may rightly ask whether ouabain had modified the response of the ouabain insensitive Na efflux to acidification Hence



a



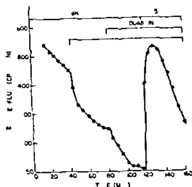
b

Fig 11 a. Sodium efflux from a barnacle fibre following acidification of the bathing medium addition of 5×10^{-5} M ouabain and restoration of the external pH to 7 8

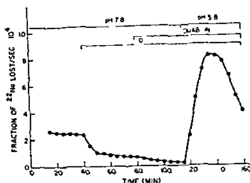
Fig 11 b The rate coefficient curve for Na efflux as recorded in this experiment

additional experiments were done The lack of effect with 5×10^{-5} M ouabain following stimulation of the Na efflux by reducing the external pH to 6 3 is illustrated by Fig 11 *a* and *b* This experiment also illustrates that a shift in pH back to 7 8 led to prompt reversal of the effect of acidification

Thus far it is quite plain that the results with ouabain cannot be readily explained if one assumes that the action of ouabain is complete But to assume this will not do On the other hand the dramatic effect produced by ouabain on the residual Na efflux into a K free solution appears to have provided the means of determining whether the relationship between the two components of the pump is reciprocal Put in another way it was no longer a question of whether the action of ouabain is complete or incomplete but rather of whether the response of the Na efflux to external acidification varies inversely with the level of activity of the ouabain sensitive Na efflux mechanism The effect on the residual Na efflux of reducing the external pH from 7 8 to 5 8 following K removal and external application of 5×10^{-5} M ouabain is illustrated in Fig 12 *a* and *b* It is seen clearly that acidification caused a most striking rise in the Na efflux and that as expected the onset of the full effect of acidification was slow (4 expts) An estimate of the rise in rate coefficient for Na efflux averaged 27 times When however the effect of acidification on Na efflux into a K free solution was measured in fibres isolated from the same muscle as that used in the preceding experiments it turned out to be considerably smaller in magnitude i.e. a 10 time rise in rate coefficient A typical experiment is recorded in Fig 13 *a* and *b* where it is also shown that external application of 5×10 M ouabain following stimulation by external acidification was without



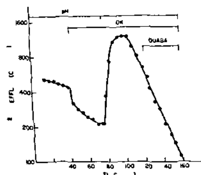
a



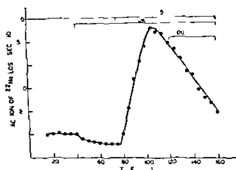
b

Fig 12 a Sodium efflux from a barnacle fibre following K removal the addition of 5×10^{5} ouabain and reducing the external pH from 7 8 to 5 8

Fig 12 b The rate coefficient curve for Na efflux as recorded in this experiment



a



b

Fig 13 a Sodium efflux from a barnacle fibre following K removal reduction in external pH from 7 8 to 5 8 and the addition of 5×10 M ouabain

Fig 13 b The rate coefficient curve for Na efflux as recorded in this experiment

effect on the Na efflux (4 expts) Failure of ouabain to affect the Na efflux suggests that activation of the second pump component is associated with de activation of the ouabain sensitive component of the Na efflux or that a low external pH interferes with the binding of ouabain to the stimulated transport system (see Fig 5 Albers Koval and Siegel 1968)

Discussion

It is generally accepted that active Na extrusion by living tissues is a process mediated by an Na K ATPase which is stopped by ouabain (Bonting 1970) The evidence as presented here is compatible with this view and confirms that the site

of action of ouabain is the external side of the plasma membrane In addition, it has been shown that ouabain concentrations as low as 10 M reduce but never stimulate the Na efflux The special significance of this observation lies in the fact that a concentration of 10^{7} M falls within the therapeutic range of cardiac glycosides

Interpretation of the wide variation in the magnitude of the effect produced by 10^{4} M or 5×10^{5} M ouabain is not so facile Variation may be an indication that the Na efflux mechanism consists of at least 2 components the activities of which under certain conditions are reciprocal Differences in the magnitude of the inhibition produced by ouabain may be directly related to changes in the internal free Ca concentration Thus for example the experiments with high K showed marked stimulation of the ouabain insensitive Na efflux. In this case the argument for increased Ca influx rests upon the evidence put forward by Hagiwara and Naka (1964) that depolarization of the fibre is associated with an increased Ca influx The fact that micro injected CaCl caused stimulation not only supports this theory but also suggests that regardless of its source any Ca reaching the sarcoplasm can bring about stimulation of the ouabain insensitive Na efflux Furthermore it will be remembered that 10^{3} M ouabain caused a diphasic effect on the Na efflux One way of explaining this result is to suppose that abolition of the ouabain sensitive Na efflux is associated with a rise in the internal Ca concentration

It is necessary to enquire into the reason for the extrasensitivity to ouabain of fibres with rate constants which persisted as a constant In keeping with this particular finding, injections of CaCl into such fibres have been found to cause stimulation of the ouabain insensitive Na efflux than in fibres with a gradual fall off in the rate coefficient (Bittar E E unpublished results) The important estion then is whether the internal Ca concentration regulates the rate at which Na is exchanged or sequestered behind the inner membranes It is a matter of common knowledge that both sarcoplasmic reticulum and mitochondria are the principal Ca sequestering organelles A reason then for the differences in ouabain sensitivity observed in the present experiments could be that the amount of free internal Na present in barnacle fibres depends on the concentration of the internal free Ca" which in turn depends on the efficacy of the sequestering mechanisms

The effect of external acidification on the Na efflux proved to be most pronounced following omission of external K ions and poisoning of the fibre with ouabain From these experiments two salient points emerge First that the onset of full action by external acidification was as before very slow This suggests that the tran port system inactivated by ouabain and the component responding to the change in external pH, are not the same Secondly the enormity of the effect caused by acidification suggests the involvement of a large surface area of the plasma membrane Together these kinetic considerations point to the T system as the location of the second pump component

Finally, since stimulation of the ouabain insensitive Na efflux also occurs upon raising the internal Ca concentration it would seem dangerous at this stage of the

work to regard this second pump component as a single system Although one is tempted to consider Na H exchange following membrane conformation as the basis of the result obtained by external acidification stimulation of the Na efflux is not typical of the exchange diffusion system as postulated by Ussing (1948) In fact it must be said that on the Ussing theory inhibition rather than stimulation would be predicted The idea that Na Ca exchange as found in squid axons (see for example Baker *et al* 1969) may be involved in this process of stimulation by a low external pH is attractive since there is evidence coming from recent work that Ca removal from the bathing medium reduces the response of the Na efflux to external acidification (Bittar E E Chen S S Danielson B G and Tong E Y unpublished results) Whether or not this second pump component is driven by metabolic energy is the subject of further investigation

This work was supported in part by funds from the Medical School Research Committee the Graduate School Research Committee the Wisconsin Heart Association the Office of Naval Research the Swedish Medical Research Council and Foreningen Margarethahemmet of Sweden.

The authors wish to thank Mr Hans Östling for indispensable technical help

B G Danielson was a Visiting Scientist of the American Heart Association during tenure of which this work was done

References

ALBERS R W G J KOVAL and G J SIEGEL Studies on the interaction of ouabain and other cardioactive steroids with sodium potassium activated adenosine triphosphatase *Molec Pharmac* 1968 *4* 324—336

BAKER P F M P BLAUSTEIN A L HODGKIN and R A STEINHARDT The influence of calcium on sodium efflux in squid axons *J Physiol* (Lond) 1969 *200* 431—458

BITTAR E E Effect of inhibitors and uncouplers on the Na pump of the Maia muscle fibre *J Physiol* (Lond) 1966 *187* 81—103

BITTAR E E P C CALDWELL and A G LOWE The efflux of sodium from single crab muscle fibres *J mar biol Ass U K* 1967 *47* 709—721

BITTAR E E S S CHEN B G DANIELSON H HARTMANN and E Y TONG An investigation of sodium transport in barnacle muscle fibres by means of the microsyringe technique *J Physiol* (Lond) 1972 *221* 389—414

BITTAR E E and E Y TONG Sensitivity of Na efflux from single barnacle muscle fibres to external H ions *Life Sci Oxford* 1971 *10* 43—48

BITTAR E E B G DANIELSON E TONG and S CHEN Sensitivity of sodium efflux in barnacle muscle fibers to the microinjection of calcium chloride *Experientia* (Basel) 1971 *27* 1432—1433

BONTING S L Sodium potassium activated adenosinetriphosphatase and cation transport In *Membranes and Ion Transport* vol 1 ed Bittar E E 1970 pp 257—363 New York Wiley Interscience

BRINLEY F J JR Sodium and potassium fluxes in isolated barnacle muscle fibers *J gen Physiol* 1968 *51* 445—477

CALDWELL P C and R D KEYNES The effect of ouabain on the efflux of sodium from a squid giant axon *J Physiol* (Lond) 1959 *148* 8—9P

CALDWELL P C and G E WALSTER Studies on the microinjection of various substances into crab muscle fibres *J Physiol* (Lond) 1963 *169* 353—372

DANIELSON B G E E BITTAR S S CHEN and E Y TONG The actions of ouabain and diphenylhydantoin on sodium efflux in single barnacle muscle fibers *Proc XXV th Int Cong Physiol Sci* Munich July 1971

DANIELSON B G E E BITTAR S S CHEN and E Y TONG The influence of low pH high K and microinjected $CaCl_2$ on the ouabain insensitive component of sodium efflux in barnacle muscle fibers *Life Sci Oxford* 1971 b *10* 833—839

DICK D A T and E J A LEA The partition of sodium fluxes in isolated toad oocytes *J Physiol* (Lond) 1967 *191* 289—308
EDWARDS C and E J HARRIS Factors influencing the sodium movement in frog muscle with a discussion of the mechanism of sodium movement *J Physiol* (Lond) 1957 *135* 567—580
GARRAHAN P J and I M GLYNN The behaviour of the sodium pump in red cells in the absence of external potassium *J Physiol* (Lond) 1967 *192* 159—174
HAGIWARA S and K NAKA The initiation of spike potential in barnacle muscle fibers under low intracellular Ca *J gen Physiol* 1964 *48* 141—162
HODGKIN A L and R D KEYNES Experiments on the injection of substances into squid giant axons by means of a microsyringe *J Physiol* (Lond) 1956 *131* 592—616
HOFFMAN J F The red cell membrane and the transport of sodium and potassium *Amer J Med* 1966 *41* 666—680
MCCLANE T K A biphasic action of ouabain on sodium transport in the toad bladder *J Pharmac exp Ther* 1965 *148* 106—110
USSING H H The use of tracer in the study of active ion transport across animal membranes In Biological Applications of Tracer Elements *Cold Spr Harb Symp quant Biol* 1948 *13* 193—200

Acta physiol scand 1973 87 391—403
From the Department of Physiology University of Goteborg Sweden

Intramural Blood Flow and Blood Volume in the Small Intestine of the Cat as Analyzed by an Indicator-Dilution Technique

By

BJORN BIBER OVE LUNDGREN and JOAR SVANVIK

Received 21 June 1972

Abstract

BIBER B O LUNDGREN and J SVANVIK *Intramural blood flow and blood volume in the small intestine of the cat as analyzed by an indicator—dilution technique* Acta physiol scand 1973 87 391—403

The mucosal red cell and plasma flow and volume in the cat small intestine were studied at various levels of smooth muscle vascular tone by means of an indicator dilution technique ^{32}P labelled red cells and plasma particles as well as ^{198}Au labelled colloid particles were used The monitored tissue region includes when using ^{32}P most of the mucosa while only the villi were "seen" with ^{198}Au During rest (total intestinal blood flow about 25 ml/min × 100 g) mucosal plasma flow was somewhat lower than total intestinal blood flow while villous plasma flow was of the same order of magnitude Mucosal and villous plasma volumes measured about 2 0 and 1 5 ml/100 ml respectively As the intestinal vascular bed was maximally dilated (total intestinal blood flow 200—250 ml/min × 100 g) by an i a infusion of isopropylnoradrenaline villous plasma flow increased to 275 ml/min × 100 ml tissue while mucosal blood flow was the same as total intestinal flow Concomitantly mucosal and villous plasma volumes increased to 3 0 and 4 0 ml/100 ml respectively Mean transit time through the villous hairpin loop decreased from 5—6 s at rest to about 1 s at maximal vasodilatation

An indicator dilution was described in a preceding paper (Biber *et al* 1973) for studying intestinal hemodynamics particularly the mucosal blood flow The method involves a close i a injection of radioactively labelled blood cells or plasma colloids and their detection with detectors sensitive to β radiation placed in the lumen and outside the intestine at the antimesenteric border This technique made it possible to analyse quantitatively mucosal blood flow and blood volume as well as the muscularis blood flow and blood volume in qualitative terms

In the present paper results will be presented obtained during resting conditions and during graded infusions of isopropylnoradrenaline a drug known to relax both the vascular and intestinal smooth muscles The experiments were performed in a manner similar to that of Kampp and Lundgren (1968) who used the inert gas

washout technique to study intestinal blood flow distribution, making it possible to compare the results obtained with the two techniques

The vascular bed of the intestinal mucosa represents one of the major circuits in the cat The experiments described below utilizing a tracer with weak β radiation (^{198}Au labelled plasma colloids) provide quantitative information concerning the capillary flow in the intestinal villi at various levels of vascular tone Such information is of considerable interest when studying e g the relationship between intestinal blood flow and absorption Furthermore the data regarding linear flow velocity and mean transit time are of particular functional interest since a countercurrent exchanger appears to exist in the intestinal mucosa (see e g Lundgren 1967)

Methods

The experiments were performed on cats anesthetized i v with chloralose (50—70 mg/kg b w) deprived of food for at least 24 h and without obvious signs of intestinal infection The theoretical background and the operative procedures of the present study were described fully in a preceding paper (Biber et al 1973) This paper also gives full details regarding the calculations of regional plasma and erythrocyte flows and volumes

To summarize briefly the method involves a continuous registration of venous outflow from an isolated intestinal segment by means of an optical drop recorder unit The transit of i a injected labelled red cells (^{32}P) or plasma colloids (^{32}P or ^{198}Au) is monitored by one detector (G M tube or semiconductor tube) in the lumen of the gut and in experiments with ^{32}P with one G M tube at the antimesenteric border Knowing the total tracer amount injected and the total blood flow mucosal plasma or red cell flows can be estimated from the height of the recorded indicator-dilution curve as earlier described (Biber et al 1973) Furthermore regional plasma or erythrocyte volumes can be determined from the area under the registered curve

To induce graded vasodilations constant i a infusions of isopropylnoradrenaline were made via a thin catheter in a small branch of the superior mesenteric artery The solution containing μg of the drug per ml saline was administered at a rate of 0 1—0 5 ml/min

Results

A Mean transit time of plasma and red cells

The transit of labelled blood particles was monitored by one detector placed in the lumen of gut (below called *internal detector*) and one placed outside the intestine at the antimesenteric border (below named *external detector*)

1 Internal detector The mean transit time ($\bar{t}_{MH}$) calculated as proposed by Zierler (1962) was determined for ^{32}P labelled red cells and plasma particles at different levels of vascular tone 13 expts in which both tracer solutions were injected were performed and the left panel of Fig 1 shows that mean transit time for both labelled solutions becomes shorter the higher the blood flow Further at corresponding blood flows $\bar{t}_{MH}$ for plasma is longer than that for the erythrocytes in agreement with the well established fact of axial streaming of blood cells

The results obtained with ^{198}Au labelled plasma colloids are illustrated in Fig 2 (lower curve) As a comparison the transit times of ^{32}P labelled plasma registred by the internal and external detectors are also included The difference in plasma

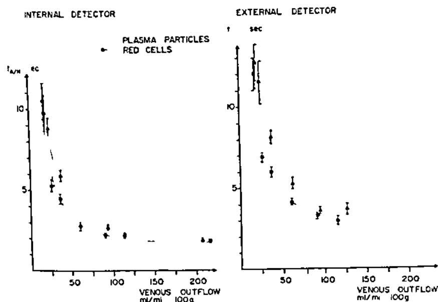


Fig 1 Cumulated data (13 expts) on the relationship between total intestinal blood flow and mean transit time ($\bar{t}_{A/H}$) for ^{32}P labelled red cells and plasma particles The transit of the tracer in the intestinal wall was monitored by internal and external detectors Vasodilatations were induced by i a infusions of isopropylnoradrenaline 4 injections of the tracer were made at every flow level in each cat Lines drawn by inspection Bars indicate ± S E Note that $\bar{t}_{A/H}$ for plasma particles is longer than that for red cells reflecting the axial streaming of blood cells

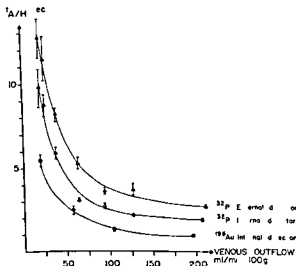


Fig 2 Cumulated data on the relation hip between total in te tinal blood flow and mean transit time ($\bar{t}_{A/H}$) for plasma particles labelled ith ^{32}P or ^{198}Au The transit of ^{198}Au particles was monitored by an internal detector during rest in 13 expts (52 injections) and n 6 of those (50 inject ons) also during hyperemia. The ^{32}P curves are identical to those of Fig 1 Vasod latations were induced by i.a. infus ons of isopropylnor adrenaline Line drawn by n pection Bars nd cate ± S E

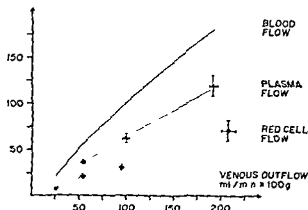


Fig 3 The relationship between total intestinal blood flow and mucosal plasma and red cell flow as determined with ^{32}P labelled red cells and plasma particles The plasma and red cell curves are each based on 84 injections made in 7 expts The blood flow curve was obtained by adding the mean values of the two other curves Mean arterial hematocrit determined from the experiments of each point varied between 30 and 37 per cent Vasodilatations were induced by i a isopropylnoradrenaline Lines drawn by inspection Bars indicate ±S E

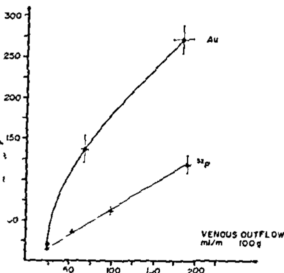


Fig 4 The relationship between total intestinal blood flow and regional plasma flow in the mucosa as determined with ^{198}Au and ^{32}P labelled plasma particles ^{198}Au labelled particles were injected during "rest" in 13 expts (52 injections) and in 6 of these (50 injections) hyperemia was induced by isopropylnoradrenaline The ^{32}P expts are identical to those of Fig 3 Lines drawn by inspection Bars indicate ± S E Note that ^{198}Au plasma flow ("villous flow) is greater than ^{32}P plasma flow (mucosal flow) at all levels of venous outflow

$t_{1/2}$ observed for ^{32}P and ^{198}Au was expected on grounds of the very low tissue penetration of the ^{198}Au β radiation as compared to ^{32}P (see Biber *et al* 1973) Thus mean transit time for ^{198}Au labelled plasma colloid particles reflects only the transit time through the vascular loops of the villi while also deeper mucosal layers are "seen" by the detector when ^{32}P is used

2 *External detector* Due to the difficulties inherent in registering the weak ^{198}Au β-radiation with the thickwalled Geiger Muller tube mean transit time through the outer layers of the intestinal wall could be measured only with ^{32}P labelled blood

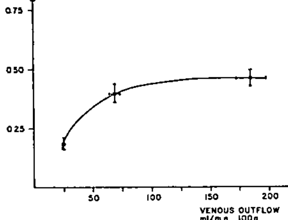


Fig 5 The relationship between total intestinal blood flow and the fraction of intramural plasma flow distributed to the villi The curve is based on the flow values of Fig 4 obtained with ^{198}Au labelled plasma particles Flow in the villi was assumed to be homogeneous and villous length was set to 0 7 mm Lines drawn by inspection. Bars indicate ± S E

particles The results shown on the right panel of Fig 1 are in principle similar to those reported for the internal detector (Fig 1 left panel) although mean transit time through the outer wall layers seems somewhat longer than that through the inner ones at comparable total venous outflows (see also Fig 2)

B Regional erythrocyte plasma and blood flows

From the indicator dilution curves recorded by the internal detector red cell and plasma flows could be determined from the height of the curve as previously described (Biber *et al* 1973) The results obtained with the ^{32}P labelled intravascular tracers (mucosal flow) are illustrated on Fig 3 By adding plasma and red cell flows it was possible to estimate also the blood flow through the monitored region When total intestinal blood flow is increased by isopropylnoradrenaline mucosal plasma and red cell flows increase as well and when total blood flow exceeds 50 ml/min × 100 g the mucosal blood flow appears to approximate that of the total venous outflow

Fig 4 illustrates the results from the experiments using ^{198}Au labelled plasma particles (villous flow) As a comparison the corresponding curve for ^{32}P labelled plasma particles is also shown At corresponding levels of vascular tone villous plasma flow as determined with ^{198}Au labelled particles appears to be larger than that of P labelled colloids (mucosal plasma flow) particularly in the higher range of venous outflows The results of Fig 4 strongly suggest that the tissue region monitored with ^{32}P labelled plasma colloids contains a less vascularized area evidently located in deeper mucosal parts This conclusion is in accordance with some observations reported in the preceding paper (Biber *et al* 1973)

Knowing plasma flow in the villi it is possible to calculate the fraction of the total intestinal plasma flow that is distributed to this region if the volume (weight) fraction of villi is known This could be calculated from the photographs of the

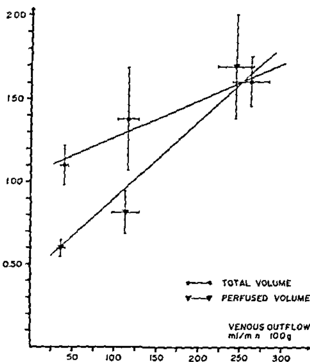


Fig 6 Cumulated data (9 expts) on the relationship between total intestinal blood flow and mucosal total and perfused red cell volumes as determined with ^{32}P labelled red cells When determin ing perfused red cell volume 4 injections were made at every flow level in each cat Mean arterial hematocrit of the ex periments at each point varied between 32 and 36 per cent. Lines constructed by the method of least squares Bars indicate ± S E Note the closely grouped values during maximal hyperemia.

transversely cut intestine performed in each experiment where correction was made or the small lumen space between the villi Fig 5 illustrates the results obtained if t e length of the capillarized part of villi was set to 0 7 mm An increasing propor tion of total plasma flow was distributed to these superficial mucosal parts as intestinal blood flow was augmented

C Regional erythrocyte plasma and blood volumes

The regional content of erythrocytes and plasma and hence of blood in the inner intestinal layers was determined with 2 different techniques (see Biber *et al* 1973) *In one of these techniques named slug injection method the regional* erythrocyte and plasma volumes were estimated from the area under the indicator dilution curve This volume is below called perfused volume since it probably represents the volume contained only in those vessels that are traversed by the injected slug (see below) The other technique named equilibration method involves an equilibra tion for about 10 min within the total blood volume of an intravascularly injected tracer (see Biber *et al* 1973) This volume is below called total volume since most vessels have probably here become labelled and are therefore included in the determination

It was possible to determine total intravascular volume only for ^{32}P labelled erythrocytes The volume values obtained with the equilibration technique using

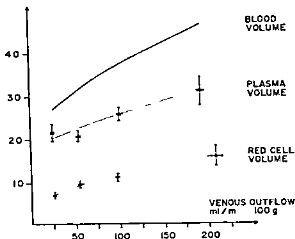


Fig 7 Cumulated data on the relationship between total intestinal blood flow and plasma and red cell volume as determined with ^{32}P labelled red cells and plasma particles The curves are based on the same expts as in Fig 3 The blood volume curve was obtained by addition of the mean values of the two other curves Mean arterial hematocrit determined from the experiments of each point varied between 30 and 37 per cent Lines drawn by inspection Bars indicate ± S E

plasma colloid particles were obviously too high probably due to the tendency of the colloid to be trapped in the reticuloendothelial cells of the intestinal mucosa In Fig 6 the two mucosal erythrocyte volumes are compared as based on 9 expts in which the volume in one and the same experiment was determined with both methods at different levels of blood flow In the lower flow range the total volume is significantly higher than the perfused one while there is no difference during intense vasodilatation in agreement with the view presented above concerning the difference between perfused and total red cell volumes As blood flow increases an increasing proportion of the total regional volume becomes perfused and the comparatively small increase of total volume may predominantly reflect a dilatation of mucosal veins

Fig 7 illustrates the correlation between total venous outflow from the intestine and the mucosal red cell and plasma volumes as determined with ^{3} P labelled blood from the area under the indicator dilution curves (perfused volume) Adding these two volumes mucosal blood volume could be estimated as shown on Fig 7 With increasing blood flow there is a concomitant augmentation of all intravascular volumes

In Fig 8 a comparison is made between the regional plasma volumes as determined with the two tracers used As total venous outflow exceeds 40—50 ml/min ×100 g villous plasma volume determined with ^{198}Au labelled plasma particles was larger than the mucosal one determined with ^{3} P labelled plasma colloids

D Regional intestinal hematocrit

The experiments utilizing ^{3} P labelled particles made it possible to estimate hematocrit in the monitored tissue region Thus regional hematocrit could be determined both from blood flow (flow hematocrit) and from blood volume (volume

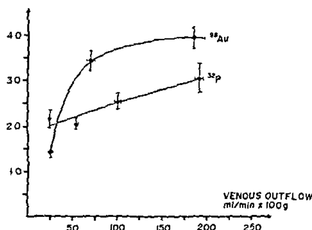


Fig 8 The relationship between total intestinal blood flow and regional plasma volume as determined by ^{198}Au and ^{32}P labelled plasma particles The ^{198}Au and ^{32}P expts are identical to those of Fig 4 and Fig 7 respectively Mean arterial hematocrit determined from the experiments of each point varied between 33 and 36 per cent Bars indicate ± S E

hematocrit) values the results being summarized in Table I At maximal vasodilatation it was sometimes difficult to make the injections of the red cell and plasma labels at similar total blood flow levels Experiments where blood flow differed by more than 20 per cent from the lowest flow level were not included in calculating the values of Table I

Discussion

Every vascular bed consists of a number of series coupled (consecutive) vascular sections i e resistance vessels exchange vessels and capacitance vessels (see e g Mellander 1960 Folkow 1967) Plethysmographic and gravimetric techniques have made it possible to study continuously and simultaneously the reactions within these vascular sections as investigated also in the small intestine of the cat (e g Folkow et al 1963) and in the dog (e g Johnson and Hanson 1962) In the lastmentioned studies however the whole organ was studied and no attempts were made to investigate separately e g the mucosal vessels The indicator dilution technique used in the present study makes it possible to follow separately the reactions within the mucosal resistance and capacitance vessels and to a certain extent also in the exchange vessels

The *resistance function* of the mucosal vessels was reflected in the regional blood flow measurements as performed with ^{32}P labelled or ^{198}Au labelled blood particles A comparison between the results obtained with these two intravascular tracers strongly suggests that blood flow within the intestinal mucosa is not homogeneous (see Fig 4) the villous blood flow (measured with ^{198}Au labelled plasma) appearing to be considerably greater than that of the entire mucosa (measured with ^{32}P labelled particles) Hence its deeper parts must contain a less well perfused section

Quantitatively the villous plasma flow ranged from about 20—50 ml/min × 100 ml tissue during rest to approximately 250 ml/min × 100 ml during maximal

TABLE I Volume and flow hematocrit (hct) in the intestinal mucosa and muscularis of the cat as determined with P labelled red cells and plasma colloids at rest and during maximal vasodilatation Mean ± S E

	Arterial hematocrit	Number of experiments	Volume hct per cent	of arterial hct	Flow hct per cent	of arterial hct
Mucosa						
Rest	34.1 ± 1.5	15	25.3 ± 2.1	77.9 ± 4.5	32.1 ± 2.1	100.2 ± 5.0
Maximal dilatation	35.0 ± 2.2	7	30.3 ± 2.1	87.3 ± 4.3	30.4 ± 2.7	86.7 ± 4.6
Muscularis						
Rest	3[illegible].1 ± 1.5	15	29.4 ± 2.1	92.4 ± 5.9	35.8 ± 2.7	113 ± 9.1
Maximal dilatation	34.2 ± 2.4	6	30.7 ± 2.1	90.7 ± 4.6	33.5 ± 3.3	96.9 ± 4.4

vasodilatation If it is assumed that regional hematocrit in the villi is approximately 50 per cent of the arterial one i e around 20 per cent (Jodal and Lundgren 1970 a) villous blood flow would be about 25—60 ml/min × 100 ml at total intestinal blood flow around 25 ml/min × 100 g and about 300 ml/min × 100 ml at total flows around 200 ml/min × 100 g Mucosal blood flows as monitored by ³ P labelled blood was somewhat less than the total intestinal blood flow during rest while they approached each other at maximal dilatation It should be underlined however that flow and volume values obtained with ³ P as a tracer are from a theoretical point less reliable than those of ¹⁹⁸Au since the blood volume contained within the monitored tissue region is not homogeneously distributed (Biber *et al* 1973)

Mucosal blood flow in the small intestine has previously been studied by means of various accumulation techniques (Grim and Lindseth 1958 Rayner Mc Lean and Grim 1960 Weiner 1961 Weiner and Grim 1966 Bacaner and Beck 1964 Csernay Wolf and Varro 1965) The mucosal blood flow values obtained with diffusable tracers such as ⁴Na and DHO are considerably lower than those recorded with the present method particularly when comparing them with the values for villous blood flow as registered with ¹⁹⁸Au labelled plasma colloids This difference is in all probability explained by the partial exclusion of ⁴Na ‑PO₄ and DHO from the mucosa due to their shortcircuiting in the intestinal countercurrent exchanger (Lundgren 1967 Kampp and Lundgren 1968) The technique utilizing labelled microspheres (Grim and Lindseth 1958) is however not liable to such drawbacks and these values agree fairly well with the present ones using ³ P labelled blood

In earlier experiments attempts were made to investigate the blood flow distribution in the cat small intestine by means of the inert gas wash out technique using ⁸Kr (Kampp and Lundgren 1968 Kampp *et al* 1968) The compartment analysis of the ⁸⁵Kr elimination curves is here complicated by the existence of the counter current exchanger since the easily diffusable inert gas is to a certain extent excluded from the villi Furthermore the analysis of the wash out curves was performed on the assumption that blood flow was fairly homogeneous in the superficial layers of the mucosa (see above) Nevertheless the mucosal flow values obtained

with the ^{85}Kr method are of the same order as those reported in the present study In the lower flow range the blood flow values reported by Kampp and Lundgren (1968) agree well with those presently obtained with ^{198}Au as corrected for hematocrit At higher blood flows the ^{85}Kr wash out technique gave values corresponding to those obtained with ^{32}P in the present study

The experiments performed with the inert gas wash out technique suggested that an extremely well vascularized area existed in deeper mucosal parts possibly associated with the cell renewal occurring at the bases of the crypts (Lundgren 1967) Thus, the intestinal mucosa of the cat seems to be rather heterogeneously perfused with a high blood flow in the villi and also in the mucosal region adjacent to the submucosa while the tissue between these two areas is less vascularized

The *capacitance* of the mucosal vessels reflected in regional blood red cell and plasma volumes as recorded by the ^{32}P labelled particles is enhanced with increasing total intestinal blood flows due to the opening up of capillaries and the dilatation of other vessels seen by the detectors (Fig 7) The values obtained for the perfused intravascular volumes in the present study coincide fairly well with those earlier reported by Jodal and Lundgren (1970 b) They are however considerably lower than the blood volume values reported by Folkow *et al* (1963) for the entire small intestine and its mesentery (7—9 ml/100 g tissue) but this included also the large veins draining the intestine

The lower perfused plasma volume observed in the ^{32}P expts when intestinal blood flow exceeded 40—50 ml/min × 100 g as compared to the ^{198}Au expts might at least partly be explained by the plasma skimming mechanism proposed by Jodal and Lundgren (1970 a) Such a mechanism would divert a proportionally greater plasma flow to the villi which is the only region monitored in the ^{198}Au expts The comparatively low plasma volume as monitored with ^{32}P labelled plasma particles also suggests that the submucosal vascular network is not registered with any high efficiency by the internal G—M tube since this would contain 20—25 ml of blood per 100 g tissue according to Jodal and Lundgren (1970 b)

According to the classical studies of the vascular archhitechture in the intestinal villi of the cat each villus is supplied by a single tortuous vessel running centrally without branching Close to the tip of the villus this ascending vessel arborizes into a dense subepithelial capillary network which drains into veins at the villous base Thus the cat villi consist almost exclusively of capillaries and changes in its intravascular volume must hence predominantly reflect the alterations in the capillary volume Since the villi were the only intestinal structures monitored in the ^{198}Au expts the absolute value and alterations in the ^{198}Au plasma volumes probably preferentially mirror alterations in capillary plasma volume and hence reflects the *precapillary sphincter activity* of the villous vascular bed (*cf* Mellander 1960) To judge from the experiments of the present study the capillary plasma volume reflecting indirectly the capillary surface area increases on an average 2—3 times when total intestinal blood flow increases 10 fold This augmentation of capillary surface area in the villi is of the same order of magnitude as in skeletal muscle and

TABLE II Calculated capillary length surface area and filtration coefficients of the intestinal villous vessels The corresponding values for skeletal muscle given by Landis and Pappenheimer (1963) are also shown as a comparison

	Intestinal villous vessels		Skeletal muscle
	Rest	Maximal vasodilatation	
Capillary length m/100 g	36000	98000	28000
Capillary area m²/100 g	0 90	2 45	0 40
Capillary filtration coefficient ml/(min × 100 g × mm Hg)	0 17	0 89	0 012
ml/(s × cm H₂O × cm² capillary surface area)	23 × 10⁻	45 × 10⁻	25 × 10⁻

in small intestine as a whole where capillary area increases fourfold upon maximal vasodilatation (Cobbold *et al* 1963 Folkow *et al* 1963)

Another indirect measure of precapillary sphincter activity in the mucosal vessels is illustrated in Fig 6 showing the difference between total and perfused red cell volumes at various levels of total intestinal blood flow As discussed above this difference is probably explained by vessels mainly capillaries which are not perfused when determining the red cell volume with the slug injection method (Biber *et al* 1973) The increase of capillary red cell volume and hence of capillary surface area is at least twofold according to Fig 6 It may be argued that an insufficient mixing of injected tracer particles would be responsible for the difference in calculated perfused and total volumes The maximal blood flow rate during isopropylnoradrenaline infusion could then constitute better mixing conditions and explain the coincident values of perfused and total volume at this state However an increased perfused plasma volume is also seen during reduced arterial inflow pressure as measured with ^{198}Au particles (Lundgren and Svanvik to be published) which seems to rule out any mixing artifact

As already stated the tissue region monitored when using ^{198}Au labelled plasma consists almost entirely of capillaries Based on the assumption that the capillary plasma volume at resting blood flow (venous outflow 25 ml/min × 100 g) is 1 4 ml/100 g tissue (Fig 8) and that hematocrit of the villous vessels amounts to 20 per cent (*cf* Jodal and Lundgren 1970 a) capillary blood volume in the villi can be calculated to be 1 8 ml/100 g tissue It is thus possible to obtain a value for capillary length and total surface area in the intestinal villi at rest A corresponding value can also be calculated for maximal vasodilatation (venous outflow 200—250 ml/min × 100 g) A capillary diameter of 8 μm was assumed and the obtained calculated values are given in Table II

Furthermore it can be estimated that the villous blood content at maximal vasodilatation would correspond to a villus (outer diameter 150 μm epithelial height 30 μm) having a central vessel surrounded by 13 capillaries running parallel to the ascending vessel In such a hypothetical villus 40 per cent of the subepithelial

surface is covered by capillaries This calculated figure is in good agreement with the values given in the morphological literature (Krogh 1929)

The capillary filtration coefficient (CFC) of the intestinal mucosa has been estimated assuming that CFC of the muscularis and the submucosa equals that of skeletal muscle (Folkow *et al* 1963) and that the mucosa comprises 45 per cent of the total intestinal weight (*cf* Jodal and Lundgren 1970 b) Values of 0 17 and 0 89 ml/min × 100 g × mm Hg are obtained at rest and during maximal vasodilatation respectively (Table II) Knowing capillary surface area and CFC a capillary filtration coefficient expressed as capillary fluid transfer rate per unit pressure difference and capillary surface area can be estimated It is evident from the calculated figures given in Table II that different values are obtained at rest and at maximal vasodilatation probably reflecting the uncertainties in the basic assumptions of the abovementioned calculations One implicit prerequisite is for example that the intestinal mucosa is homogeneously perfused by blood which evidently is not the case (see above) An alternative explanation to the recorded difference may be a widening effect of the drug on the capillary pores although no other observation supports this hypothesis It is however quite clear that the fenestrated mucosal capillaries have a higher water conductivity per unit surface area than the continuous capillaries in cat skeletal muscle as evident from Table II which also includes data from skeletal muscle (Landis and Pappenheimer 1963) probably obtained at maximal vascular smooth muscle relaxation Table II also shows that the intestinal mucosa is more vascularized than the skeletal muscle as reflected in the size of capillary length and surface area

The difference in mean transit time noted between red cells and plasma (Fig 1) is in agreement with classical concepts of velocity gradients of laminar flow in blood vessels It was also shown that the transit of ^{198}Au labelled plasma was shorter than that of ^{32}P labelled plasma in all probability due to the much smaller tissue volume monitored when using ^{198}Au To judge from anatomical data the blood volume contained within the capillary network is about 10 times greater than that in the central villous vessel (Krogh 1929 see above) *i e* linear flow velocity in the villous capillaries is approximately 1/10 of that in the ascending vessel Hence mean transit time in the capillary network is at least 4 sec at the lower end of the flow range studied corresponding to a linear velocity of 0 15—0 20 mm/s if a villus is assumed to be 0 7 mm long At maximal vasodilatation linear flow rate amounts to around 0 7 mm/s

These calculated values for linear flow rates in the mucosal capillaries are comparatively low (*cf* Folkow and Neil 1971) and are of particular functional importance since a countercurrent exchanger exists in the intestinal mucosa of the cat The low linear flow rates of blood must imply that the efficiency of the countercurrent exchanger is high when it is approached from the mucosal side thus effectively damping the rate of entrance into the portal blood stream of easily diffusible solutes

It was proposed by Jodal and Lundgren (1970 a) that plasma skimming occurred

in the intestinal wall leading to a hematocrit in the villus vessels of only about 20 per cent at arterial hematocrits around 32—33 per cent The data on volume hematocrit reported in this study (Table I) also suggest that the villous blood has a low red cell content although not quite as low as the values given by Jodal and Lundgren The present results thus seem to support the plasma skimming hypothesis but they do not give any clue as to the localization of the high hematocrit vessels

This research has been sponsored by grants from the Swedish Medical Research Council (No B 72 14X 2855 03 and partly by B 72 14X 16 08A) from the Faculty of Medicine University of Goteborg and from Svenska Sallskapet for Medicinsk Forskning
AB Vitrum generously supplied us with heparin Thanks are also due to AB Pharmacia for its supply of Rheomacrodex® and Macrodex®

Referencee

BACANER M B and J S BECK Regional blood flow measurement in vivo for a definable geometry *Amer J Physiol* 1964 *206* 962—966
BIBER B O LUNDGREN L STAGE and J SVANVIK An indicator-dilution method for studying intestinal hemodynamics in the cat *Acta physiol scand* 1973 In press
COBBOLD A B FOLKOW I KJELLMER and S MELLANDER Nervous and local chemical control of pre capillary sphincters in skeletal muscle as measured by changes in filtration coefficient *Acta physiol scand* 1963 *57* 180—192
CSERNAY L F WOLF and V VARRO Der Kreislaufgradient im Dunndarm *Z Gastroenterologie* 1965 *3* 261—265
FOLKOW B Regional adjustments of intestinal blood flow *Gastroenterology* 1967 *52* 423—432
FOLKOW B O LUNDGREN and I WALLENTIN Studies on the relationship between flow resistance capillary filtration coefficient and regional blood volume in the intestine of the cat *Acta physiol scand* 1963 *57* 270—283
FOLKOW B and E NEIL *Circulation* Oxford university press New York 1971
GRIM E and E O LINDSETH Distribution of blood flow to the tissues of the small intestine of the dog *Univ Minn med B ll* 1958 *30* 138—145
JODAL M and O LUNDGREN Plasma skimming in the intestinal tract *Acta physiol scand* 1970 a *80* 50—60
JODAL M and O LUNDGREN Regional distribution of red cells plasma and blood volume in the intestinal wall of the cat *Acta physiol scand* 1970 b *80* 533—537
JOHNSON P C and K M HANSON Effect of arterial pressure on arterial and venous resistance of intestine *J appl Physiol* 1962 *17* 503—508
KAMPP M and O LUNDGREN Blood flow and flow distribution in the small intestine of the cat as analysed by the Kr85 wash out technique *Acta phys l scand* 1968 *72* 282—297
KAMPP M O LUNDGREN and J SJOSTRAND The distribution of intravascularly administered lipid soluble and lipid insoluble substances in the mucosa and the submucosa of the small intestine of the cat *Acta physiol scand* 1968 *72* 469—480
KROGH A *The Anatomy and Physiology of Capillaries* New Haven Yale Univ Press 1929
LANDIS E M and J R PAPPENHEIMER Exchange of substances through the capillary walls *Handbook of Physiol* 1963 Sect 2 Circulation Vol II 961—1034
LUNDGREN O Studies on blood flow distribution and counte current exchange in the small intestine of the cat *Acta phy l scand* 1967 Suppl 303
MELLANDER S Comparative studies on the adrenergic neuro-hormonal control of resistance and capacitance blood vessels in the cat *Acta physiol scand* 1960 *50* Suppl 1 6
RAYNER R L MACLEAN and E GRIM Intestinal tissue blood flow in shock due to endotoxin *Circul t Res* 1960 *8* 1212—1217
WEINER, D *Kinetics of distribution of D₂O in the tissues of the canine ileum* (Thesis) Minneapolis Univ of Minnesota 1961 Quoted from Grim E The flow of blood in the mesenteric vein *Handbook of Physiology* Sect 2 Vol II 1963 1439—1456
WEINER D E and E GRIM Kinetics of distribution of D₂O in canine intestinal tissues *Amer J Physiol* 1966 *211* 600—606
ZIERLER K L Equations for measuring blood flow by external monitoring of radioisotopes *Circulat Res* 1965 *16* 309—321

Acta physiol scand 1973 87 404—410

From the Institute for Experimental Medical Research University of Oslo and Medical Dept VII Ullevål Hospital Oslo Norway

Nervous and Humoral Control of Vascular Resistance during Acute Hemorrhagic Hypotension in Rabbits

By

H Aars and S Akre

Received 22 June 1972

Abstract

Aars H and S Akre *Nervous and humoral control of vascular resistance during acute hemorrhagic hypotension in rabbits* Acta physiol scand 1973 87 404—410

Peripheral vascular resistance in anesthetized rabbits quickly increased during hemorrhagic hypotension despite almost unchanged sympathetic nerve activity Simultaneous measurement of blood flow to denervated and innervated kidneys showed that the acute rise of renal vascular resistance during hypotension was independent of innervation In other animals the left innervated kidney was perfused with blood from either the host or a donor rabbit by means of a pump Renal vascular resistance invariably rose during systemic hypotension when the kidney was perfused with blood from the rabbit subjected to hemorrhage but not when perfused with blood from a normovolemic animal The results support the view that increased peripheral vascular resistance during the first minutes of hemorrhagic hypotension is caused by humoral agents rather than by increased sympathetic activity

A vasoconstrictive response to reduction of arterial blood pressure may be brought about by humoral and nervous mechanisms either combined or separately The nervously mediated vasoconstriction is generally believed to be due to a rise of sympathetic activity caused by withdrawal of the inhibitory influence of baroreceptor activity on sympathetic neurons (Scher 1965) In anesthetized cats dogs and rabbits however investigations have shown that activity in cardiac splanchnic and renal sympathetic nerves may remain largely unaltered during the early stages of hemorrhagic hypotension (Kezdi and Geller 1968 Thamer Weidinger and Kirchner 1969 Aars and Akre 1971) The results imply that peripheral vasoconstriction if present under these circumstances would be mediated by humoral pressor agents. The present study was therefore undertaken to establish whether peripheral vascular resistance was elevated by acute hemorrhagic hypotension in rabbits showing little or no increase of activity in renal and splanchnic nerves and if so to examine the relative role of humoral and direct nervous effects in the vasoconstrictive response Peripheral vascular resistance was calculated from arterial pressure and blood flow

in the descending thoracic aorta renal vascular resistance was determined when blood pressure was reduced in rabbits with one denervated and one innervated kidney and when the kidney of a rabbit subjected to hypotension was perfused with blood from either the same or another normovolemic animal

Materials and Methods

The investigations were carried out in 20 adult rabbits anesthetized with a mixture of 1 % chloralose 3 ml/kg and 25 % urethane 3 ml/kg (Aars and Akre 1968) Body weights ranged from 3 to 5 4 kg The animals were tracheotomized but respiration was only assisted when in 5 animals an electromagnetic flowmeter probe (Nycotron) was positioned on the descending thoracic aorta. Thorax was closed and the respirator disconnected before proceeding with the experiment Unless otherwise stated all rabbits respired air

Arterial blood pressure was measured with a Statham transducer connected to a catheter in the right femoral or common carotid artery Pressure was reduced by removing blood from the right jugular vein or through the arterial catheter in the course of 30 s to 2 min and in steps of 10 ml Each hemorrhagic level was maintained for 30 s to 1 min occasionally 2 min and total duration of most hemorrhagic periods was 3—7 min In the denervation experiments hypotension could last up to 16 min Blood clotting was prevented by heparin

In the five rabbits with a flowmeter probe on descending aorta peripheral vascular resistance was calculated from the relationship between mean aortic blood flow and mean arterial pressure The same procedure was followed for the kidneys when in five other rabbits flowmeter probes (Nycotron i d 1—2 mm) were placed on both renal arteries Shortly before the left kidneys had been surgically denervated and the stripped renal vessels bathed in 1 % lidocaine Innervation of the right kidneys remained intact

Renal perfusion experiments were performed in five pairs of rabbits The left renal artery in the recipient animal was connected to an occlusion pump set at a constant flow Blood was delivered to the pump from a catheter in the right common carotid artery of either the recipient (auto-perfusion) or donor animal (cross-perfusion) and the renal venous outflow was returned to the right jugular vein of the animal whose blood was then perfusing the kidney Transit time from the carotid artery to the kidney was 2—4 min depending on the rate of flow Perfusion pressure was measured with a transducer connected to the circuit close to the kidney The kidney was kept moist and warm by covers and a heating lamp

In experiments with aortic flow or renal perfusion sympathetic activity was differentially recorded in the intact left renal nerve The activity was rectified and integrated and the output from the integrator was recorded together with the neurogram on a jet ink writer (Elema Mingograph) After subtraction of electrical noise nervous activity was calculated from the integrator curves as per cent of activity at resting control blood pressure (Aars and Akre 1968)

Results

Aortic flow In the rabbits with a flowmeter probe on thoracic aorta peripheral vascular resistance was unchanged or decreased by withdrawing 10 ml of blood At subsequent steps of bleeding resistance increased in four animals and remained unaffected in one The increase appeared 2—4 min after start of bleeding at a time when removal of 20 ml of blood had reduced mean arterial blood pressure to 47—55 mm Hg (individual reductions of 15—25 mm Hg) When 30—40 ml of blood had been removed vascular resistance ranged from 100—583 % of control levels (mean 319 %) As in earlier experiments (Aars and Akre 1971) activity in the renal nerve was not consistently changed by the blood pressure reduction The activity disappeared after sectioning of the nerve proximal to the recording site demonstrating the efferent direction of previously measured impulses

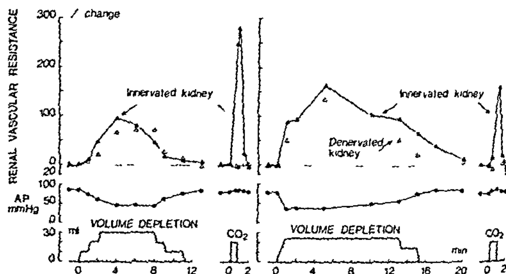


Fig 1 Effects of blood volume depletions on mean arterial pressure and vascular resistance in denervated and innervated kidney of a rabbit weighing 4 2 kg Completeness of denervation and integrity of innervation demonstrated by response to respiration of 20 % CO_2 in air

Denervated and innervated kidneys Renal vascular resistance usually rose in response to hemorrhage in both denervated and innervated kidneys A typical example is shown in Fig 1 In this rabbit renal resistance started to increase after removal of only 10 ml of blood at a blood volume reduction of 30 ml resistance had risen 70– 100 % from control values In the second run a more abrupt removal of blood caused the pressure to drop to lower levels and resistance then rose by 140– % Furthermore and most important — there were no major differences between changes of vascular resistance in the denervated and innervated kidneys Complete denervation and intact innervation respectively were repeatedly demonstrated by the response to inhalation of 20 % CO_2 in air for 10– 30 s

A rise of renal vascular resistance in response to hemorrhage was observed in 9 of 11 expts In 7 of these expts vascular resistance rose in both denervated and innervated kidneys Similarly when in 2 expts resistance remained unaltered during hemorrhage this was the case in both kidneys On two occasions resistance rose only in the innervated kidney The denervation was checked by the response of the renal vasculature to exposing the rabbits to 20 % CO_2 in air This procedure always resulted in marked increases of vascular resistance in innervated kidneys with no or insignificant changes in denervated kidneys At control mean blood pressures (60– 100 mean 80 mm Hg renal blood flow was 10–71 mean 30 ml min and 6–42 (mean 17 ml min in denervated and innervated kidneys respectively

Renal perfusion Two typical examples of renal perfusion are illustrated in Fig 2 During auto-perfusion Fig 2 A reduction of blood pressure was followed by a fall

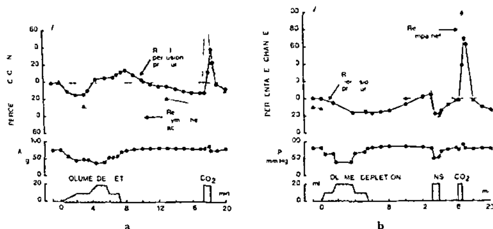


Fig 2 Effects of blood volume depletion on mean arterial pressure renal sympathetic nerve activity and renal perfusion pressure The kidney perfused at constant flow so that changes in perfusion pressure indicate changes in vascular resistance Perfusion pressure is plotted as per cent change from control values in each experiment sympathetic activity as per cent changes from control at the beginning of the experiment shown in Fig 2 A The kidney was alternately perfused with blood from the host animal (auto perfusion Fig 2 A) and a donor animal (cross perfusion Fig 2 B) After restoration of blood volume the host rabbit was subjected to respiration of 20 % CO_2 in air or to electrical stimulation of the left aortic nerve (ANS 100 Hz 0 1 ms 6 V)

in sympathetic nerve activity and initially decreased perfusion pressure The reduced perfusion pressure reflected the decrease in renal sympathetic activity because due to the tubing nervous and humoral effects on renal vascular resistance in this experiment were separated by 2 min The increase in perfusion pressure after about 3 1/2 min at a time when sympathetic activity was still falling could have been caused by pressor substances released into the systemic circulation about 1 1/2 min after onset of bleeding *i e* before the 10 ml of blood had been completely removed The integrity of the sympathetic nervous system was demosntrated by the marked increase in renal nerve activity—and the parallel rise of renal vascular resistance—during inspiration of 20 % CO in air

When in the same animal the kidney was afterwards perfused with blood from the normovolemic donor animal at the same rate of flow the bleeding had much the same initial depressive effect on renal sympathetic activity and perfusion pressure as during auto perfusion (Fig 2 B) On this occasion however humoral agents from the depleted animal could not reach the renal vasculature and perfusion pressure remained low throughout the period of volume depletion The responses to subsequent electrical stimulation of the recipient s left aortic nerve (ANS Fig 2 B) and exposure to 20 % CO in air illustrated the range of control of renal vascular resistance exerted by the sympathetic nervous system

Cross perfusion of the kidney was successfully carried out eight times and auto-perfusion seven times in the five rabbits with flow rates of 4—8 ml/min Control

mean blood pressure of the recipient rabbits ranged from 60 to 95 mm Hg Control mean perfusion pressures were 60 to 138 mm Hg (average 98 mm Hg) During hemorrhage and auto perfusion of the kidney renal vascular resistance rose exceeding control levels in all rabbits The increase corresponded to the release of vasoconstrictive substances beginning 30 s to 3 min after start of hemorrhage (absolute figures time lag subtracted) Maximum increase varied from 5 to 44 % of vascular resistance at control pressure In contrast renal perfusion pressure usually decreased —never increased—when the kidney of the depleted rabbit was perfused with blood from the normovolemic donor animal In two cases of cross perfusion blood pressure was reduced in the donor animal but not in the recipient renal perfusion pressure then increased in the same way as for auto perfusion and bleeding of the recipient animal In most instances renal nerve activity varied only slightly during the hemorrhagic periods showing no consistent differences between periods of auto- and cross perfusion of the kidney

Discussion

The first set of experiments indicated that peripheral vascular resistance increased within a few minutes of hemorrhagic hypotension in the anesthetized rabbits while as before (Aars and Akre 1971) renal nerve activity was largely unchanged This implies either that renal nerves and vasculature did not participate in the net vasoconstriction or that the vasoconstriction was mainly due to the action of humoral pressor agents

Studies of renal vascular resistance confirmed participation by the vascular bed of the kidney in the overall pressor response However in general the response was not dependent on intact innervation qualitatively it was the same in denervated and innervated kidneys This result which indicates the action of humoral factors was confirmed in perfusion experiments Renal vascular resistance rose only when the kidney was perfused with blood from the rabbit subjected to hemorrhagic hypotension

The recorded changes of renal vascular resistance in response to hypotension should be considered in a qualitative rather than a quantitative sense For the denervated and innervated kidneys this is due to the large differences in flow and possibly in sensitivity to pressor agents Furthermore at very low blood flow minor errors in flow measurements will drastically influence the calculation of vascular resistance In the perfusion experiments the resistance was abnormally high probably due to mechanical effects of the pump perfusion Even so the perfused kidneys had retained considerable vascular sensitivity to humoral and nervous stimuli and the results supported the conclusion of a release of pressor agents in response to hemorrhagic hypotension

It may be calculated that the pressor substances were released after removal of only 4—8 % of total blood volume (based on 58 ml/kg Knorpp and Pixberg 1960) In the present studies no attempts were made to determine the nature of these

pressor substances but others have shown that catecholamines (see Chien 1967) vasopressin (Rocha e Silva and Rosenberg 1969) and renin (see Page and McCubbin 1968) are released in response to hemorrhage of the same magnitude and duration as in the present investigation Thus cutaneous vasoconstriction following hemorrhage in anesthetized dogs was unaffected by denervation but blocked by phenoxybenzamine, indicating an elevation of plasma catecholamines (Bond *et al* 1970) In cats increased splenic and intestinal vascular resistance 1 min after bleeding and reduction of mean blood pressure to about 50 mm Hg was effected by angiotensin and vasopressin Vascular constriction was maintained after denervation adrenalectomy and injection of phenoxybenzamine (Greenway and Stark 1969 McNeill Stark and Greenway 1970)

Haddy Scott and Molnar (1965) reported that in the dog forelimb vasoconstriction in response to systemic arterial hypotension was not prevented by denervation (bilateral vagotomy procainization of carotid sinuses section of local nerve supply) They concluded that humoral vasoconstrictor substances participated in the response These investigators also observed a marked rise of vascular resistance in perfused kidneys during hypotension and showed that vasoconstrictor substances were released from the kidney They made no attempt however to distinguish between nervous and humoral effects through cross perfusion or denervation of the kidneys

The importance of humoral factors in the present renal vasoconstrictive response to hemorrhage indicates that these factors also played a major role in the increase of peripheral vascular resistance found at the level of descending thoracic aorta There is thus no reason to assume different changes of sympathetic activity in kidneys and other regions during the present experiments This view is supported by the observation of almost no changes in heart rate of rabbits subjected to stepwise bleeding In 20 rabbits with control mean blood pressure of 83—110 mm Hg heart rate remained within +6 and −5 % of control when the pressure was reduced 10—40 mm Hg Average figures at 10 20 30 and 40 mm Hg below control were +0 3 +0 2 −0 8 and −0 3 % respectively (own unpublished results) Heart rate could not be measured from the present low speed pressure tracings

A rise in sympathetic nerve activity following reduction of blood pressure may result from a fall in baroreceptor discharge and/or stimulation of arterial chemoreceptors However in the present and previous studies (Aars and Akre 1971) sympathetic activity on the whole did not rise in response to hemorrhagic hypotension in rabbits Furthermore increased vascular resistance restricted to the innervated kidney in the present series was found in only 2 of 11 expts This lack of sympathetic activation during acute hypotension may be due to a depressive effect of the anesthesia on sympathetic neurons as suggested by Skovsted Price and Price (1969) who obtained similar results in cats exposed to halothane The sensitivity of the neurons to a release of inhibitory activity from baroreceptors might be reduced Alternatively the control sympathetic activity—presumably increased by the anesthesia—might be too high to allow a further rise by reducing baroreceptor activity Sympathetic activity was inversely related to changes in aortic nerve activity at

pressures above control levels, however (Aars and Akre 1971), and stimulation of chemoreceptors as well as acute barodenervation (Aars and Akre 1971) produced a reflex increase of sympathetic activity also at *low* pressures. The lack of sympathetic activation in response to acute hypotension was therefore probably due to the fact that in rabbits, arterial chemoreceptors are not activated by acute reductions of blood pressure before the pressure falls below 20 mm Hg. In cats this threshold is usually found between 40 and 100 mm Hg (Ott, Kiwull and Wiemer 1971). Our results therefore support the contention that in anesthetized animals reduction of baroreceptor activity during acute lowering of arterial pressure from normal control levels has no major effect on sympathetic activity. Even so, peripheral vascular resistance may rise markedly in the early stages of hemorrhage, primarily owing to release of humoral pressor agents.

This work was supported by J. L. Tiedemanns Tobakfabrik, Joh. H. Andresens Medical Fund, the Norwegian Council on Cardiovascular Diseases, and Anders Jahres Fund for the Promotion of Science.

References

AARS, H. and S. AKRE, Effect of angiotensin on sympathetic nerve activity. *Acta physiol. scand.* 1968. 74. 134—141.

AARS, H. and S. AKRE, Sympathetic nervous response to induced fall and rise of arterial blood pressure in anesthetized rabbits. *Pflugers Arch. ges. Physiol.* 1971. 3[illegible]6. 223—230.

BOND, R. F., G. F. LACKEY, J. A. TAXIS and H. D. GREEN, Factors governing cutaneous vasoconstriction during hemorrhage. *Amer. J. Physiol.* 1[illegible]0. 219. 1210—1[illegible]15.

CHIEN, S., Role of the sympathetic nervous system in hemorrhage. *Physiol. Rev.* 1967. 47. [illegible]—[illegible].

GREENWAY, C. V. and R. D. STARK, Vascular responses of the spleen to rapid haemorrhage in the anaesthetized cat. *J. Physiol. (Lond.)* 1963. 204. 169—1[illegible]9.

HARDY, F. J., J. B. SCOTT and J. I. MCLNAR, Mechanism of volume replacement and vascular constriction following hemorrhage. *Amer. J. Physiol.* 1965. 207. 16[illegible]—181.

KEZDI, P. and E. GELLER, Baroreceptor control of postganglionic sympathetic nerve discharge. *Amer. J. Physiol.* 1968. 214. 427—435.

KNORPP, K. and H. L. PIXBERG, Blutstromenbestimmung mit [illegible]Cr bei [illegible] Neuseeland Kaninchen. *Pflugers Arch. ges. Physiol.* 1970. 31[illegible]. 357—3[illegible].

MCNEILL, J. R., R. D. STARK and C. V. GREENWAY, Intestinal vasoconstriction after hemorrhage: [illegible] of vasopressin and angiotensin. *Amer. J. Physiol.* 1[illegible]0. 219. 134[illegible]—1347.

OTT, N., P. KIWULL and W. WIEMER, Zur Aktivierung der Chemorec[illegible] des Glomus caroticum durch [illegible] arterielle Drucksenkung bei Kaninchen und Katze. *Pflugers Arch. ges. Physiol.* 1971. 3[illegible]5. [illegible]8—3[illegible].

PAGE, I. H. and J. W. MCCUBBIN, *Renal hypertension.* Chicago, Year Book Med. Publ. 1968.

ROCHA E SILVA, M., JR. and M. R. ROSENBERG, The release of vasopressin in response to haemorrhage and its role in the mechanism of blood pressure regulation. *J. Physiol. (Lond.)* 1[illegible]. 20[illegible]. [illegible]3[illegible]—55[illegible].

SCHER, A. M., Control of arterial blood pressure. Measurement of pressure and flow. In *Physiology and Biophysics* [illegible] Ruch and H. D. Patton, pp. [illegible]. W. B. Saunders, Philadelphia and London 1[illegible].

SKOVSTED, P., M. L. PRICE and H. L. PRICE, The effects of halothane on [illegible] ganglionic sympathetic activity and [illegible] reflexes. *Anesthesiology* 1[illegible]. 31. 507—514.

THAMER, V., W. WEIDINGER and F. KIRCHNER, Sympathische Aktivität [illegible] hämorrhagischen Schock. *Z. Kreisl.-Forsch.* 1[illegible]. [illegible]8. 4[illegible]—[illegible]34.

Acta physiol scand, 1973 87 411—421

From the Departments of Aviation and Naval Medicine Faculty of Medicine Karolinska Institutet and the Department of Physiology Gymnastik och Idrottshogskolan, Stockholm Sweden

Oxygen Uptake during Maximal Work at Lowered and Raised Ambient Air Pressures

By

L. FAGRAEUS J KARLSSON D LINNARSSON and B SALTIN

Received 11 July 19 2

Abstract

FAGRAEUS L. J KARLSSON D LINNARSSON and B SALTIN *Oxygen uptake during maximal work at lowered and raised ambient air pressures* Acta physiol scand 1973 87 411—421

The maximal aerobic power ($\dot{V}_{O_2}$ max) was measured in 12 subjects exercising on a bicycle ergometer breathing air at 0 68 1 0 and 1 40 ATA ambient pressures Seven of the subjects were also investigated at 2 0 and 3 0 ATA. $\dot{V}_{O_2}$ max averaged 3 6/ l min STPD at 1 0 ATA and was reduced by 14 per cent at 0 68 ATA ($p < 0.001$) while at 1 40 ATA $\dot{V}_{O_2}$ max was increased by 9 per cent ($p < 0.001$) The increased P_{IO_2} at the two highest pressures did not result in any further improvement of $\dot{V}_{O_2}$ max compared to 1 40 ATA At all pressures changes in endurance time paralleled those of $\dot{V}_{O_2}$ max. Ventilation decreased markedly as ambient pressure increased with a concomitant rise of P_{ACO_2} up to a mean value of 43 mm Hg at 3 0 ATA as compared to 32 mm Hg at 1 0 ATA It is concluded that moderate hyperoxia produced by increased ambient air pressure enhances circulatory transport of oxygen in maximal exercise and that the working muscles have an aerobic potential exceeding what the circulation normally can offer

The changes in work performance induced by acute exposure to altitude have been thoroughly investigated (for reviews see Margaria 1967 Åstrand and Rodahl 1970 Faulkner 1971) Maximal oxygen uptake is decreased the cause being a limitation of oxygen transport at the alveolo-capillary membrane Johnson 1967 Blomqvist Johnson and Saltin 1969) and the decrease in aerobic power can be offset by restoring a normal oxygen tension (Saltin *et al* 1968 Wyndham *et al* 1971)

It has also been shown that at normal ambient pressure the maximal aerobic power and the endurance time on a supramaximal work load are increased when the inspired oxygen concentration is increased This was first observed by Hill Long and Lupton in 1924 Similar observations have been made by Nielsen and Hansen (1937) and Margaria *et al* (1961) who compared the maximal oxygen uptake breathing air with that obtained with different high oxygen concentrations at normal ambient pressure An increase of P_{IO_2} from normal to 300—700 mm Hg was observed to improve maximal oxygen uptake by 10—20 per cent The influence on the maximal oxygen uptake of the variations in ambient air pressure from 0 50 t

TABLE I. Anthropometric and functional data

Subject	Sex	Age years	Height cm	Weight kg	$\dot{V}_{O_2}$ max l min⁻¹ STPD
BL	M	25	189	76	4.35
JK	M	31	178	68	3.47
LF	M	32	189	81	3.69
DL	M	29	172	61	3.22
MH	F	25	171	56	2.16
BL	M	24	175	63	3.02
BS	M	36	187	81	5.03
KS	M	27	183	72	3.68
BR	M	30	185	81	4.39
GS	M	28	171	69	3.90
LJ	M	30	189	78	3.69
RP	M	34	184	75	3.45

atmospheres absolute (ATA) have been studied by Eagan and Plese (1969) who found a direct relationship between *ambient pressure and V_{O_2} max.* Similar results were obtained by Wyndham *et al.* (1970) in the pressure range of 0.83–1.13 ATA (P_{IO_2} = 120–170 mm Hg) where maximal aerobic power was found to vary linearly with the ambient pressure.

All the aforementioned investigations indicate that in conditions where the organism is offered an increased amount of oxygen, the maximal aerobic power is increased above normal. However, the linear relationship between maximal aerobic power and ambient pressure suggested by Wyndham and his group may seem surprising in view of the inherent nonlinear properties of the oxygen transport system. We considered it of interest, therefore, to determine the maximal aerobic power within a wider range of ambient air pressures (0.68–3.0 ATA).

Methods

Twelve healthy subjects (one female) with large variations in maximal oxygen uptake performed submaximal and maximal exercise on a mechanically braked cycle ergometer (von Döbeln 1954). For anthropometric and functional data see Table I. They were all familiar with exhaustive exercise on a cycle ergometer and were acquainted with the experimental procedure before the experiments started. Experiments were performed with the subjects breathing air in a combined altitude and high pressure chamber at pressures of 520, 760 and 1060 mm Hg (0.68, 1.0 and 1.40 ATA, respectively). Seven of the subjects were also investigated at 860, 1520 and 2280 mm Hg (1.13, 2.0 and 3.0 ATA, respectively). The temperature inside the chamber was kept between [illegible] and 23 °C throughout the experiments by means of an air conditioning system. In order to provide the subjects with air of known and constant composition during the gas exchange measurements, air from high pressure tanks outside the chamber was allowed to pass a large water bottle inside the chamber to become humidified and then via a wide bore tubing led past the inspiratory side of a low resistance breathing valve (dead space 10 ml) at a flow rate of 3.5 l [illegible] ATPS. In this way, and since the volume of the wide bore tubing distal to the point of attachment of the breathing valve exceeded 1.5 liters, no chamber air was inspired by the subject even during maximal inspiratory effort.

The pressure drop at a flow rate of 7.0 l/s BTPS on the inspiratory side of the breathing circuit amounted to 1.6 cm H_2O at 1.0 ATA, 2.5 cm H_2O at 2.0 ATA and 3.4 cm H_2O at 3.0 ATA. The corresponding pressure drops on the expiratory side were 2.7, 4.1 and 7.0 cm H_2O, respectively.

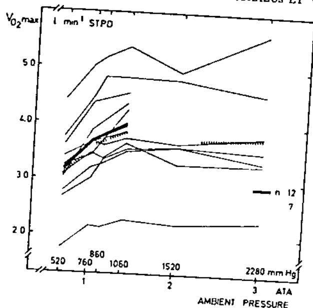


Fig 1 Maximal oxygen uptake ($\dot{V}_{O_2}$ max) at lowered normal and raised ambient air pressures (P_B 520 760 860 1060 1520 and 2280 mm Hg respectively) The thin lines show individual values while mean values are indicated by solid or broken heavy lines

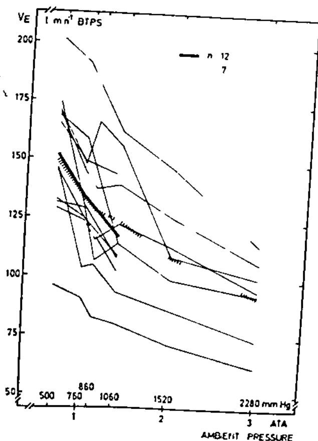


Fig 2 Pulmonary ventilation ($\dot{V}_E$) during the last 30 s of maximal exercise at lowered normal and raised ambient air pressures For further information see Fig 1

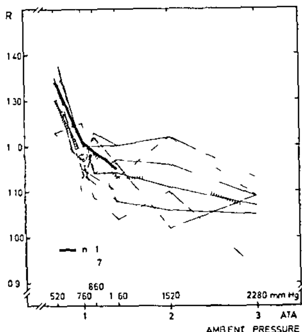


Fig 3 Respiratory exchange ratio (R) during the last 30 s of maximal exercise at lowered, normal and r ised amb ent air pressures For further information see Fig 1

with a beat to-beat rate meter (Lindborg Ödman and W gertz 1969) and corded on a pen re corder During submaximal exercise heart rate as measured as the mean value o er tl e last m nute At maximal exerc se the maximal value at the po nt of exh u tion v as taken

N trogen exchange is a potential source of error in $\dot{V}_O$ determinat on when using the Douglas b g method sub equent to changes in ambient air p essure In the present study th error has been of ins gn ficant magnitude For example when the exp red air s mpling started after 11 – 12 min at 3 0 ATA the nitrogen uptake should have been about 30 ml min STPD (*cf* Hesser 1965) caus ng an underestimation of $\dot{V}_{O_2}$ by only about 6 ml m n⁻ STPD 24 double dete minations of maximal oxygen uptake were made at var ous pres ures the nterval between the determinations var ng f om 1 to 30 days The mean error of a single determ nation as found to be 1 4 per cent nd d d not vary w th the ambient pressu e

Stag decomp ess on v as never nec s ary du to th hort xposu (?0 – 30 min)

Conv ntional statist cs have been appl d S gn ficanc of int aindi dual diff ences w s test d w th Student s t test

Results

The results are summarized in Table II and Fig 1 – 4

Maximal oxygen uptake ($\dot{V}_{O_2}$max Fig 1) An intraindividual comparison shows that $\dot{V}_{O_2}$max was significantly higher at normal ambient pressure than at 0 68 ATA mean values being 3 67 and 3 18 l min⁻ STPD respectively ($p < 0\ 001$) Similarly with a rise in ambient pressure from normal to 1 40 ATA $\dot{V}_O$ max increased signifi cantly by 9 per cent to 4 01 min⁻ STPD ($p < 0\ 001$) In the group studied at higher pressures the $\dot{V}_O$ max values at 2 0 ATA and 3 0 ATA were slightly lower than at 1 40 ATA although still 5 – 6 per cent higher ($p < 0\ 01$) than the sea level control values

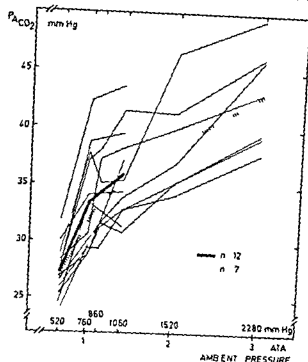


Fig. 4. Alveolar CO_2 tension ($P_{A_{CO_2}}$) during the last 30 s of maximal exercise at lowered, normal and raised ambient air pressures calculated by means of the Bohr equation using an assumed dead space to tidal volume ratio of 0.12. For further information see Fig. 1.

Increased gas density is known to seriously affect pulmonary functions (Lanphier 1969, Miller, Wangensteen and Lanphier 1971). Thus the maximal voluntary ventilation during 15 s (MVV 15) (Miles 1966) and exercise ventilation (Hesser, Fagraeus and Linnarsson 1968, Salzano, Rausch and Saltzman 1970) have been found to decrease. The progressive fall in ventilation at maximal work with increasing ambient pressure found in the present study (Fig. 2) showed largely the same exponential tendency as has been described for MVV 15 by Miles (1966) and Lanphier (1969). The ventilatory restriction imposed by high gas density prevented the hyperventilation with regard to CO_2 that normally takes place at maximal exercise. In fact CO_2 was retained as reflected by the much lower than normal respiratory exchange ratio in the hyperbaric experiments where R averaged 1.06 compared to 1.30 under sea level conditions (Fig. 3). As a consequence alveolar P_{CO_2} rose (Fig. 4) and a respiratory acidosis was developed. A similar CO_2 retention is known to occur even with submaximal exercise in hyperbaric environment (Hesser *et al.* 1968, Lanphier 1969).

Besides the increased gas density at least two other mechanisms will impede CO_2 elimination during muscular exercise in hyperbaric air. First, hyperoxia has been shown to cause part of the ventilatory decrement during submaximal exercise in hyperbaric air (Hesser *et al.* 1968) and it is probable that this mechanism plays a role at maximal exercise as well. Second, hyperoxia will interfere with the CO_2 transport from the tissues if it is great enough to bring about any substantial increase of the oxygen saturation of the venous blood from the working muscles.

Kaijser (1970) observed that both the arterial and the venous acidosis at exhaustion were more severe with 3 ATA O_2 than with 1 ATA air despite lower lactate concentrations in the hyperoxic situation The lower pH at 3 ATA O_2 was due to respiratory acidosis the arterial P_{CO_2} averaging 49 mm Hg as compared to 34 mm Hg at 1 ATA air These values are similar to the calculated $P_{A_{CO_2}}$ values found at corresponding ambient pressures in the present study where, on the other hand no differences were observed in lactate concentration of the arterialized capillary blood The hypothesis that the acidity of the muscle cell may set a limit for its performance (*cf* Kaijser 1970) is compatible with the findings of Luft Finkelstein and Elliott (1971) that even small amounts of CO_2 in the inspired air produce a significant decrease of the maximal oxygen uptake It seems possible however that the hypercapnia occurring at maximal exercise in hyperbaric environments may exert depressing influences also on other functions of importance for the performance of maximal exercise such as central circulation and psychomotor performance

Heart rate in exercise at various ambient pressures In submaximal exercise with acute exposure to hypoxia the mechanisms controlling heart rate and cardiac output seem to compensate for the decrease in arterial oxygen content thereby ensuring a constant supply of oxygen (Stenberg Ekblom and Messin 1966 Hughes *et al* 1968) An inverse relationship between heart rate at a standardized submaximal work load and P_{A_O} was found in the present study over the whole range of pressures studied and may be an expression of a similar mechanism Whether the link between heart rate and arterial oxygen content acts via the oxygenation of the tissues or has a specific chemoreflex origin is so far unclear The mechanisms predominantly responsible might be different in hypoxia normoxia and hyperoxia Apart from an oxygen dependent reduction of the heart rate (for review see Schaefer 1965) a non-oxygen dependent decrement of a similar magnitude has been found to occur both at rest and during exercise in air at 4 5 ATA (Hesser *et al* 1968) Such a non oxygen dependent mechanism may be responsible for part of the decrease in heart rate observed during submaximal exercise in the present experiments at 2 0 and 3 0 ATA

The maximal heart rate was less influenced by acute changes in ambient pressure than was the heart rate at submaximal exercise Stenberg *et al* (1966) observed no significant changes in maximal heart rate and cardiac output in subjects acutely exposed to an ambient pressure of 0 61 ATA whereas Wyndham *et al* (1970) found no changes in maximal heart rate in subjects exposed to a slightly elevated ambient pressure

The oxygen pulse (Table II) *i e* the product of stroke volume and arteriovenous O_2 difference increased with the ambient pressure in a way similar to $\dot{V}_{O_2}max$ It has been shown that the stroke volume at exhaustion is similar to sea level value at 0 61 ATA air (Stenberg *et al* 1966) and it is likely that so was the case in the present 0 68 ATA expts as well leaving a decreased a v O_2 difference to account for the decrease in oxygen pulse

Since data about the central circulation in maximal exercise in hyperbaric conditions breathing air is missing both in the present study and in the literature, it is not possible to distinguish whether the increased oxygen pulse compared to sea level control then is due to an increased a v O_2 difference an increased stroke volume or both in combination It is likely that the arterial oxygen content at 1 40 ATA compared to sea level control was increased by 4–5 per cent which alone could not account for the increase in oxygen pulse by 9 per cent As pointed out above Nielsen and Hansen (1937) have postulated that the increased oxygen content in the arterial blood during maximal work in hyperoxia positively can effect the heart which may result in an enhanced stroke volume and cardiac output If this is the case this increase in flow adds to the increase in arterially transported oxygen being offered to the tissues thereby explaining the 9 per cent increase in V_{O_2}max and oxygen pulse in the 1 40 ATA expts At 3 0 ATA however the further increase in arterial oxygen content does not seem to improve the working conditions of the heart

Subjective experiences of exhaustive exercise With increasing ambient pressure muscular fatigue of the legs became gradually less dominant as the cause to stop the maximal work task Instead respiratory discomfort became noticeable in the 2 0 and 3 0 ATA expts This together with a short lasting spell of dizziness at the end of the maximal work period made the point of exhaustion less well defined in the 3 0 ATA expts Manifestations of nitrogen narcosis were observed by Cook (1970) in 5 out of 8 subjects when performing exhaustive work at 3 0 ATA breathing 5 5 % O_2 in N_2 The dizziness experienced by some of our subjects was reported to bear a strong resemblance to the symptoms of intoxication which are characteristic of exposures to much higher nitrogen partial pressures This phenomenon might be explained by observation (cf Hesser Adolfson and Fagraeus 1971) that high CO_2 tensions enhance the narcotic effect of raised N_2 tensions

This study was supported by the Swedish Medical Research Council Projects 40X–682 and 40X–2203 The skilful technical assistance of Mrs Margareta Elgh Mr B Lennholm and Mr B Lundin is gratefully acknowledged

References

ASMUSSEN E Muscular exercise In *Handbook of Physiology Respiration* edited by W O Fenn and H Rahn Washington D C Amer Physiol Soc 1965 3 vol II 939–978

ASMUSSEN E Respiration *Acta physiol scand* 1969 Suppl 330 p 6

ASMUSSEN E and M NIELSEN Physiological dead space and alveolar gas pressures at rest and during muscular exercise *Acta physiol scand* 1956 38 1–21

ASMUSSEN E and M NIELSEN Alveolo-arterial gas exchange at rest and during work at different O tensions *Acta physiol scand* 1960 50 153–166

ÅSTRAND P O and K RODAHL *Textbook of Work Physiology* McGraw Hill Book Company New York 19 0 669 pp

BARKER S B and W H SUMMERSON The colorimetric determination of lactic acid in biol gical material *J biol Chem* 1941 *138* 535 554

BLOMQVIST G R I JOHNSON Jr and B SALTIN Pulmonary diffusing capacity limiting human performance at altitude *Acta phy ol s a d* 1969 (84–287

COOK, J C Work capacity in hyperbaric environments without hyperoxia *Aerospace Med* 19 0 *41* 1133–1135

DOBELN VON W A simple bicycle ergometer *J appl Physiol* 1954 7 2 2 4

EAGAN C J and E R FLESE Cardiorespiratory and metabolic effects of work during hyperand hyperbaria *Fed Proc* 1969 *8* 593

FAULKNER, J A Maximum exercise at medium altitude In *Frontiers of Fitness* edited by R J Shephard, Charles C Thomas Publ Springfield Illinois USA 1971 chapt 18 360–3 5

HESSER C M Breath hold ng under high pressure In *Ph s olog v of B eath Hold During and the Ama of Japan* edited by H Rahn and T Yokoyama Natl Acad Sci — Natl Res Counc l Publ 1341 1965 165—181

HESSER C M J ADOLFSON and L FAGRAEUS Role of CO in compressed air narcosis *Aero face Med* 1971 *42* 163—168

HESSER, C M L FAGRAEUS and D LINNARSSON Cardio-resp ratory responses to exerc se in hyperbaric environment *Report Lab Aviat Naval Med Ka ol Inst Sto kh lm* Aug 1968 10 pp

HILL A. V C N H LONG and H LUPTON Muscular exercise lactic acid and the supply and utilisation of oxygen *Proc roy Soc B* 1924 *97* 155—176

HOLMGREN A and H LINDERHOLM Oxygen and carbon dioxide tensions of arterial blood during heavy and exhaustive exercise *Acta ph siol scand* 1958 *44* 203—215

HUGHES, R. L M CLODE R. H T EDWARDS T J GOODWIN and N L. JONES Effect of insp red O on cardiopulmonary and metabolic responses to exercise in man *J appl Physiol* 1968 *24* 336—347

JOHNSON R L. JR Pulmonary diffusion as a limiting factor in exercise stress *C culat Res* 1967 *20* 154—160

KAIJSER L Limiting factors for aerobic muscle performance *A ta physiol sca d* 1970 Suppl 346 1—96

LANPHIER E H Pulmonary function In *The Ph siology and Medicine of Diving* edited by P B Bennett and D H Elliott Balliere Tindall and Cassel London 1969 58—112

LILIENTHAL J L JR R L RILEY D D PROEMMEL and R E FRANKE An experimental analysis in man of the oxygen pressure gradient from alveolar air to arterial blood during rest and exercise at sea level and at alt tude *Amer J Ph siol* 1946 *147* 199—216

LINDBORG B TH ÖDMAN and O WIGERTZ A beat to-beat heart rate meter with linear an log output for muscular exercise studies *Rep rt Lab Aviat Naval Med Karol Inst Stockholm* Dec 1969 23 pp

LUFT U C S FINKELSTEIN and J C ELLIOTT Resp ratory gas exchange acid base balance and elect olytes during and after maximal work breathing 15 mm Hg P_{ICO} To be publ shed in CO and Metabolic Regulations *Proc Satellite Symp XXV I t Co gr Phys l Sci* Monte Carlo 1971

MARGARIA R. *Ex rcise at Altitude* Excerpta M d ca Foundation Amsterdam 1967 216 pp

MARGARIA R H T EDWARDS and D B DILL, The possible m chanisms of contracting and paying the oxygen debt and the role of lact c acid in muscular contraction *Amer J Physiol* 1933 *106* 689—715

MARGARIA R P CERRETELLI S MARCHI and L ROSSI Maximum exercise in oxygen *Int Z a gew Phy iol* 1961 *18* 465—467

MILES S *Underwat r Med ine* Staples Press London. 1966 332 pp

MILLER J N O D WANGENSTEEN and E H LANPHIER Ventilatory l m tations on exertion at depth *Proc Fo th S mposi m on Underwater Physiolog* edited by C J Lambertsen Academic Press New York and London 1971 317—323

NIELSEN M and O HANSEN Maximal korpe liche Arbe t bei Atmung O e cher Luft *Skandinav Archiv Ph ol* 1937 *76* 37—59

OTIS A. B Quantitative relationships n steady state ga exchange In *Handbook f Physiology R p at o* edited by W O F nn and H Rahn Wa h ngton D C Amer Physiol Soc 1964 3 vol I 681—698

SALTIN B R F GROVER C G BLOMQVIST L H HARTLEY and R L JOHNSON JR Max mal oxygen uptake and cardiac output after 2 we ks at 4300 m *J ppl Ph s l* 1968 *25* 400—409

SALZANO J D C RAUSCH and H A SALTZMAN C rd o esp atory responses to exe cise at a simulated seawater d pth of 1000 feet *J ppl Phys ol* 19 0 *28* 34—41

SCHAEFER K E Circulatory adaptation to th r quir ments of life under mo than one atmosphere of p essure In *Handbook of Physiol g Ci l t n* edited by W F Ham lton and P Dow Wash ng ton D C Amer Phys ol Soc 1965 2 vol III 1843—1873

STENBERG J B EKBLO I and R MESSIN Hemodyn m c response to work at s mulated altitude 4000 m *J appl Ph iol* 1966 *21* 1589—1594

STROM G The nfluence of anoxia n lactat utilization n man afte prolonged mu cula work *Act ph i l scand* 1949 *17* 440—451

WYNDHAM C H R KOK N B STRYDOM G G ROGERS and S ZWI Phys olog cal effe ts of acute changes n alt tude n a d p m n *J appl Phy iol* 1971 *30* 232—237

WYNDHAM C H N B STRYDOM A J VAN RENSBURG and G G ROGERS Effects on maximal oxygen ntake of acute changes in altitude in a de p m ne *J ppl Phy iol* 1970 *9* 552—555

Acta physiol scand 1973 87 422—424

From the Department of Anatomy and Embryology, College of Veterinary Medicine
Helsinki Finland

Incorporation of ^{35}S-Labelled Cysteine in the Subcommissural Organ of the Rat after Adrenalectomy and after Treatment with Hydrocortisone

By

U ATTILA and S TALANTI

The physiological role of the subcommissural organ (SCO) is still unclarified although numerous hypotheses have been presented (see for references Palkovits 1965) Morphological evidence speaks in favour of a secretory function especially of the subcommissural ependyma The cytoplasmic selectively stainable material in the ependymal cells "secretion" can be demonstrated by the methods which also stain the hypothalamic neurosecretory material This "secretion" appears to be rich in histochemically demonstrable cystine (Talanti 1958)

A correlation between the function of the SCO and the adrenal glands has also been experimentally proved Palkovits (1965) reported changes in the secretion of aldosterone after destruction of the SCO and he observed histoquantitative changes in the zona glomerulosa after administration of SCO extract

An attempt has been made to employ the intensity of incorporation of labelled as an indicator of the secretory or metabolic activity of the SCO (Talanti 1971) It is likely that a high proportion of cysteine is incorporated in proteins and at least partly in the "secretion" of the SCO The present study constitutes a part of the studies concerning the influence of the function of the endocrine glands on the SCO Its principal aim was to investigate the effect of experimental changes in the function of the adrenal glands on the incorporation of labelled cysteine in the SCO Changes were induced in the adrenal glands by adrenalectomy and by treatment with hydrocortisone

54 male adult albino rats were used All rats received a standard pellet diet and tap water *ad libitum* 18 of the animals were adrenalectomized under anesthesia another 18 rats were treated with hydrocortisone (Hydro-Adreson Organon) at 5 mg/daily i m and 18 rats served as controls After 14 days counted from adrenalectomy or from the beginning of hydrocortisone treatment all animals received an average dose of 150 μCi of ^{35}S labelled cysteine (L-Cysteine S 35) hydrochloride The Radiochemical Centre Amersham Bucks England) by i p injection 6 animals from each group were sacrificed by rapid decapitation at each of the times 45 min 4 h and 24 h after the cysteine injection

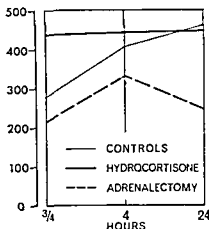


Fig 1 Diagrams showing the grain counts (mean of 6 animals) found at different times after injection of the isotope in the ependyma of the subcommissural organ

The brains were fixed in 10% formalin embedded in paraffin and sectioned sagittally at 10 μm For autoradiography the mounted sections were dipped in Kodak NTB 2 emulsion The slides were stored in light tight boxes for 30 days at 4° C and subsequently developed in Kodak D 11 developer and fixed with Kodak rapid fixer The staining of the sections was made with hematoxylin eosin

Grain counts were made using an eye piece micrometer with ×10 eye piece and ×100 objective The area counted measured 150 μm×75 μm Ten areas of the subcommissural ependyma of each rat were counted From the series of counts ob tained the mean values and their standard deviations were calculated

The results are presented in Fig 1 Intense incorporation of labelled cysteine was seen in the ependyma of the SCO of the rats in the control group The counts are in agreement with previous studies (Sloper Arnott and King 1960 Talanti 1968 and 1971) The standard deviations of all means were in order of 5—10 units Adrenalectomy as well as treatment with hydrocortisone exerted a distinct effect on the uptake of labelled cysteine In the groups killed 45 min and 4 h after the injec tion of labelled cysteine the animals treated with hydrocortisone showed clearly higher counts than were obtained with the controls while the counts of the adrenal ectomized rats were slightly lower In the animals killed after 24 h the incorporation of the label was quite low in the group of adrenalectomized animals The rats treated with hydrocortisone also showed a tendency of lower incorporation than those of the control group at this time

In lack of observations at more closely spaced time intervals little can be said con cerning the rates of uptake as a function of time It seems however as if the gradual uptake which obviously took place throughout the period of observation in the con trols had been replaced by early massive uptake under cortisone effect the level reached very soon after injection persisting virtually unchanged up to 24 h In the adrenalectomized animals too the maximum rate of uptake seems to have occurred very early and the depletion of labelled material after 4 h is strikingly evident

Acta physiol scand 1973 87 422—424

From the Department of Anatomy and Embryology College of Veterinary Medicine
Helsinki Finland

Incorporation of ³⁵S-Labelled Cysteine in the Subcommissural Organ of the Rat after Adrenalectomy and after Treatment with Hydrocortisone

By

U Attila and S Talanti

The physiological role of the subcommissural organ (SCO) is still unclarified although numerous hypotheses have been presented (see for references Palkovits 1965) Morphological evidence speaks in favour of a secretory function especially of the subcommissural ependyma The cytoplasmic selectively stainable material in the ependymal cells secretion can be demonstrated by the methods which also stain the hypothalamic neurosecretory material This secretion appears to be rich in histochemically demonstrable cystine (Talanti 1958)

A correlation between the function of the SCO and the adrenal glands has also been experimentally proved Palkovits (1965) reported changes in the secretion of aldosterone after destruction of the SCO and he observed histoquantitative changes in the zona glomerulosa after administration of SCO extract

An attempt has been made to employ the intensity of incorporation of labelled ³⁵S-cysteine as an indicator of the secretory or metabolic activity of the SCO (Talanti 1971) It is likely that a high proportion of cysteine is incorporated in proteins and at least partly in the secretion of the SCO The present study constitutes a part of the studies concerning the influence of the function of the endocrine glands on the SCO Its principal aim was to investigate the effect of experimental changes in the function of the adrenal glands on the incorporation of labelled cysteine in the SCO Changes were induced in the adrenal glands by adrenalectomy and by treatment with hydrocortisone

54 male adult albino rats were used All rats received a standard pellet diet and tap water *ad libitum* 18 of the animals were adrenalectomized under anesthesia another 18 rats were treated with hydrocortisone (Hydro-Adreson Organon) at 5 mg/daily i m and 18 rats served as controls After 14 days counted from adrenalectomy or from the beginning of hydrocortisone treatment all animals received an average dose of 150 μCi of ³⁵S labelled cysteine (L-Cysteine S 35 hydrochloride The Radiochemical Centre Amersham Bucks England) by i p injection 6 animals from each group were sacrificed by rapid decapitation at each of the times 45 min 4 h and 24 h after the cysteine injection

Acta physiol scand 1973 87 425—427
From the Zoophysiological Laboratory B University of Copenhagen Denmark

Two Types of Excitatory Activity Recorded from the Median Giant Fiber of the Earthworm

By

G R. J CHRISTOFFERSEN and LEE A MILLER*

The median giant fiber one of three dorsal septate giant fibers in the earthworm *Lumbricus terrestris* is one element in an efferent afferent synaptic circuit producing after discharge and repetitive firing (Kao 1956 1960 Kao and Grundfest 1957) In this communication we report cyclic non fatiguing potentials that produce repetitive firing in the median giant fiber to a single short stimulus On the other hand small depolarizations occurring only on the repolarizing phase of the action potential exhibit rapid fatigue to repetitive stimulation and are unable to reexcite the median giant fiber

The nerve cord was removed following short term anesthesia in 15 % ethyl alcohol and *immediately placed in an aerated saline solution* (Kao and Grundfest 1957) Stimuli were presented to the rostral end of the nerve cord via platinum *electrodes or intracellularly to the median giant fiber via a glass microelectrode* Electrical recordings were made with two intracellular glass microelectrodes and *with a suction electrode at the caudal end The average resting potential was* -73 mV (range -64 to -83 mV) and the average spike amplitude was 87 mV (range 70 to 106 mV) No spontaneous activity was recorded with microelectrodes in the median giant fiber and action potentials in the lateral giant fibers produced only slight hyperpolarizations that lasted 1 ms and did not exceed 0 5 mV when recorded in the median giant fiber The results were based on 24 preparations and experiments were carried out at 19 to 21° C Preparations were viable for up to 9 h

Extra action potentials (after discharge) and repetitive firing appeared in 22 preparations in response to a paired intracellular or extracellular stimulus where the second action potential fell within the relative refractory period of the first In 6 preparations a single intracellular or extracellular stimulus was sufficient to produce this activity Repetitive firing could last for up to 1 s and have frequencies as high as 400 spikes/s The mechanism responsible for the generation of repetitive action potentials at least at lower frequencies is shown in Fig 1 a Here repetitive firing in response to a single intracellular stimulus is preceded by depolarizing potentials having identical shapes from cycle to cycle showing no potentiation or fatigue The repetitive firing can be broken should the depolarizing potentials fail to produce an action potential (not shown in Fig 1 a) Consequently the action potential itself

* Biologisk Institut Odense Universitet, Odense DK 5000 Denmark

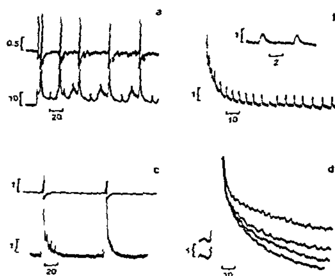


Fig 1 Intracellular (lower traces) and extracellular (upper traces) recordings from the median giant fiber and the whole nerve cord of the earthworm
(a) A single stimulus produced by current injection for 3 ms elicits a series of identical depolarizing potentials and repetitive firing Spike peaks are off scale and spike amplitudes are greater than 100 mV
(b) Low amplitude pulses appear on the repolarizing phase of the action potential The insert shows a portion of the trace expanded and indicates that the pulses have a duration of about 1.5 ms and an amplitude of 1 mV
(c) These low amplitude fatigue rapidly and are not visible on the falling phase of a second action potential occurring 100 ms after the first.
(d) This multiexposure oscillogram shows the summated form of these subthreshold potentials and the sequence to four consecutive stimuli 200 ms apart The summations can raise the repolarizing phase of the action potential by as much as 4 mV All stimuli except that in Fig 1 a, are at threshold of the median giant fiber Vertical scales are in mV and horizontal scales in ms

serves as the stimulus for the next cycle of depolarizing potentials A neural circuit involving synaptic contact between the median giant fiber and the giant interneurons i each segment (Gunther and Walther 1971) may provide the morphological basis or repetitive activity An ephaptic mechanism is mainly responsible for the production of circus activity and repetitive responses recorded from crayfish lateral giant axons (Watanabe and Grundfest 1961 Kusano and Grundfest 1965) The contribution of ephaptic pathways involving circus activity in the earthworm remains to be clarified

Depolarizing potentials that always remain subthreshold appeared on the repolarizing phase of the action potential in the median giant fiber in 17 preparations (Fig b—d) This activity which is previously unreported could appear as a series of distinct pulses lasting up to 300 ms with the first discernible pulse appearing 5 ms after an intracellularly or extracellularly initiated action potential (Fig 1 b) The maximum amplitude of a pulse was 1 mV and the duration was from 1.5 to 2 ms (insert of Fig 1 b However this activity appeared more often in a summated form where the single pulses became less distinct and the repolarizing phase of the action potential could be raised by as much as 4 mV as seen in the upper sweep of Fig 1 d Most importantly these low amplitude depolarizing potentials were characterized by remarkably rapid fatigue to repetitive stimulation (Fig 1 c d) in contrast to depolarizations responsible for repetitive firing (Fig 1 a Fatigue could occur within 100 ms as illustrated in Fig 1 c where the pulses seen on the re

polarizing phase of the first action potential disappear on the second action potential In Fig 1 d (a multiexposure oscillogram) the amount of summated activity declines progressively to four consecutive action potentials at 200 ms intervals The recovery of this activity increases gradually and reaches the non fatigued level with in a few seconds There was no indication of facilitation and unlike the depolarizing potentials producing after discharge and repetitive firing they were incapable of reexciting the giant fiber They could not be elicited by subthreshold stimuli nor were they observed to occur spontaneously The mechanism responsible for the production of this activity is unknown although it may represent an after depolarization similar to that reported by Yamagishi and Grundfest (1971) for the crayfish medial giant axon Another possibility may involve a synaptic mechanism at the septum itself (Antonov 1964) The septum functions as an electrotonic junction for the through conducted action potential (Kao and Grundfest 1957) but small vesicles are regularly spaced along the septal membranes in each segment of the giant fiber of the earthworm *Eisenia foetida* (Hama 1959) (The presence of such vesicles however could not be confirmed in *L terrestris* (Coggeshall 1965) The feasibility of electrotonic and chemical mechanisms coexisting at the septum in the median giant fiber of the earthworm is now under investigation

One of us (L A M) was supported in part by a Biomedical Sciences Support Grant No 5 SO5 RRO7114 04 National Institutes of Health USA

References

ANTONOV V F The effect of monoiodoacetate and temperature on the conduction of a nerve impulse through the septa of the earthworm giant axons *Biophysics* 1964 9 194—203

COGGESHALL R E A fine structural analysis of the ventral nerve cord and associated sheath of *Lumbricus terrestris* L *J comp Neurol* 1965 *125* 393—438

GUNTHER, J and J B WALTHER Funktionelle Anatomie der dorsalen Riesenfaser System von *Lumbricus terestris* L (Annelidia Oligochaeta) *Z morph Tiere* 1971 *70* 253—280

HAMA K Some observations on the fine structure of the giant nerve fiber of the earthworm *Eisenia foetida* *J biophys biochem Cytol* 1959 6 61—66

KAO C Y Basis for after discharge in the median giant axon of the earthworm *Science* 1956 *123* 803

KAO C Y Postsynaptic electrogenesis in septate giant axons II Comparison of medial and lateral giant axons of crayfish *J Neurophys* *1* 1960 23 618—635

KAO C Y and H GRUNDFEST Postsynaptic electrogenesis in septate giant axons I Earthworm median giant axon *J Neurophys* *1* 1957 *20* 553—573

KUSANO K and H GRUNDFEST Circus reexcitation as a cause of repetitive activity in crayfish lateral giant axons *J cell comp Physiol* 1965 *65* 325—336

YAMAGISHI S and H GRUNDFEST Contribution of various ions to the resting and action potentials of crayfish medial giant axons *J Membrane Biol* 1971 5 345—365

WATANABE A and H GRUNDFEST Impulse propagation at the septal and commissural junctions of crayfish lateral giant axon *J gen Physiol* 1961 45 267—308

Acta physiol. scand 1973 87 428—430

From the Department of Physiology Karolinska Institutet Stockholm

Kinetics of Secretion of Sympathetic Neurotransmitter as a Function of External Calcium Mechanism of Inhibitory Effect of Prostaglandin E

By

LENNART STJÄRNE

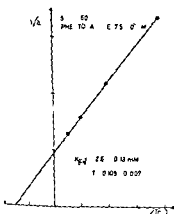
In the present study kinetic methods were applied to characterize the calcium dependence (cf Kirpekar and Misu 1967) of nerve stimulation induced secretion of tracer noradrenaline (NA) from the sympathetic nerves of isolated guinea pig vas deferens and to define kinetically the calcium dependence of the inhibitory effect of exogenous prostaglandin E_2 (PGE$_2$) on NA secretion

The experiments were carried out in 20 guinea pigs weighing 250—300 g as described elsewhere (Stjärne 1973 a) The superfusion medium was Tyrode solution in which the calcium concentration was varied from 1 to 8 mM Desipramine 6×10^{-7} M and normetanephrine 10^{-5} M were added to the medium to block local rebinding of released NA and phentolamine 7.5×10^{-5} M to release NA secretion from α adrenoreceptor dependent negative feed back restriction of NA secretion Stjärne 1973 b) In some experiments the tissue was also preincubated with 5 8 11 14-eicosatetraynoic acid (ETA) 3×10^{-5} M for 10 min to block local formation of endogenous PGE NA secretion was induced by field stimulation with biphasic pulses of supramaximal strength and a duration of 1 5 ms Trains of 300 stimuli were applied at 5 Hz with about 10 min intervals

The fractional secretion of tracer NA was calculated as the ratio of the nerve stimulation induced rise in total efflux of ^3H and the total ^3H content of the tissue at the time of stimulation To characterize the calcium dependence of NA secretion kinetically the reciprocal of fractional tracer NA secretion was plotted according to Lineweaver and Burk against the reciprocal of the calcium concentration

Fig 1 which gives the average of 4 expts shows that the apparent K_m of the secretory process was 2.61 ± 0.013 mM the closeness of this value to the normal calcium concentration of tissue fluid speaks in favour of the validity of the method. The maximum fractional NA secretion at this concentration of α adrenoceptor blocking drug which brings about nearly maximal disinhibition of NA secretion (Stjärne 1973 c) was 0.105 ± 0.007 for 300 pulses at 5Hz thus implies an average of 3.5×10^{-4} of the total store of tracer NA per nerve impulse

In the study of the effect of PGE$_2$ on the calcium dependence of NA secretion the tissue was preincubated with ETA to suppress endogenous PGE formation Under these conditions it has previously been found that the inhibitory effect of exogenous PGE$_2$ is inversely related to the calcium concentration of the medium (Stjärne 1973 d) The present results which were plotted (Fig 2 each point represents an average of 3—4 observations) according to Eadie and Hofstee clearly



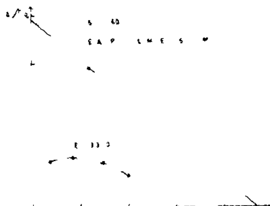


Fig 1 Lineweaver Burk plot of calcium dependence of tracer NA secretion For explanation see Text

Fig 2. Plot according to Eadie and Hofstee of calcium dependence of tracer NA secretion and of the inhibitory effect of PGE$_2$

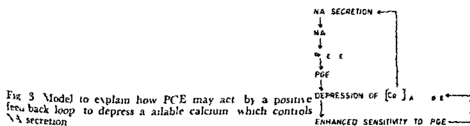


Fig 3 Model to explain how PGE may act by a positive feed back loop to depress available calcium which controls NA secretion

show that exogenous PGE 3 3×10^{-9} M depresses the maximum secretion of tracer NA (intercept with abscissa) Moreover as expected from the above mentioned results PGE depressed the apparent affinity for calcium of the secretory mechanism (slope of curve gives $-1/K_c$) progressively more with falling calcium concentration in the medium

Since under the experimental conditions calcium appears to play the decisive role in determining NA secretion the present results suggest that the mechanism of the inhibitory effect of PGE on NA secretion may well consist in depression of the apparent affinity for calcium of the secretory mechanism The results may also help to clarify the tremendous inhibitory potency of PGE for which there is no known receptor on NA secretion the progressively increasing PGE induced depression of apparent affinity for calcium of the secretory mechanism with falling external calcium is consistent with the possibility that PGE operates a positive feed back loop multiplying the initial reduction in available calcium (Fig 3)

It is also conceivable that this is the PGE mediated mechanism whereby NA secreted from sympathetic nerves restricts further secretion of NA. The released transmitter may via probably neural α adrenoceptors (Stjärne 1973 b) trigger the release of small amounts of possibly neural (Stjärne 1972) endogenous PGE to operate a positive feed back loop leading to drastic reduction in calcium available for the process of transmitter secretion lasting for as long as the NA concentration around the specific α adrenoceptors remains sufficiently high (Fig 3)

This work was supported by a grant from the Swedish Medical Research Council (project B73-04X-30-09). I want to thank my wife Mrs. Eivor Stjärne for excellent technical assistance.

References

KIRPEKAR S M and Y MISU Release of noradrenaline by splenic nerve stimulation and its dependence on calcium *J Physiol (Lond)* 1967 *188* 219–234

STJÄRNE L Prostaglandin E restricting noradrenaline secretion neural in origin *Acta physiol scand* 1972 *86* 574–576

STJÄRNE L Lack of correlation between profiles of transmitter efflux and of muscular contraction in response to nerve stimulation in isolated guinea-pig vas deferens *Acta physiol scand* 1973 a In press

STJÄRNE L Dual alpha adrenoceptor mediated control of secretion of sympathetic neurotransmitter one mechanism dependent and one independent of prostaglandin E *Prostaglandins* 1973 b In press

STJÄRNE L Uncompetitive character of inhibition by prostaglandin E_2 of the enhancing effect of α blocking drugs on noradrenaline secretion in isolated guinea-pig vas deferens *Acta physiol scand* [illegible] 1973 c In press

STJÄRNE L Inhibitory effect of prostaglandin E_2 on noradrenaline secretion from sympathetic nerves as a function of external calcium *Prostaglandins* 1973 d In press

Acta physiol scand 1973 87 431—432

From the Institute of Aviation Medicine Royal Norwegian Air Force Blindern Oslo Norway
and Radiological Department Ullevål Hospital Oslo Norway

Ventricular Dilatation in the Diving Seal

By

ARNOLDUS SCHYTTE BLIX and RAGNAR HOL

During diving the cardiac output of seals is known to decrease as a result of a conspicuous bradycardia combined with a constant stroke volume (Elsner *et al* 1964) Also it is known that the oxygen content of the arterial blood diminishes linearly from 22 to about 5 vol % during a 15 min dive (Scholander 1940) Thus insufficient oxygenation of the heart of the seal during a prolonged dive is imminent and although an elevated myocardial oxygen extraction during the increased diastolic periods (10—20 s) must be considered this animal may provide an interesting model for the study of coronary insufficiency in man

The present preliminary report deals with some additional information on the functional properties of the heart of the diving seal obtained during an angiographic examination of a Harp seal (*Pagophilus groenlandicus*) during 10—18 min dives using the method described by Blix *et al* (1973)

As shown in Fig 1 the right ventricle of the heart was strikingly dilated with a large end systolic volume already after only 6 min of submersion This picture closely resembles that of humans during acute cardiac infarction

This result supports those of Ferrante and Opdyke (1969) who found evidence for a 25—50 % decrease in left ventricular contractility during a 5 min dive in the Nutria Likewise it agrees with Kjekshus *et al* (1971) who stated that ventricular dilatation represents the most important compensatory mechanism in the maintainance of cardiac output and the external ventricular performance during acute coronary insufficiency in the dog

Thus contrary to man where the above described effects indicates severe cardiac failure the reversible ventricular dilatation observed in the seal may represent an important life securing mechanism in this diving species



Fig 1 Angiocardiograms of the seal after 6 min of submersion (prone position contrast medium injected into the right atrium) LEFT Atrial systole showing the filling of the dilated right ventricle (arrow) RIGHT Early diastole showing the huge ventricular residual volume (lower arrow) and the filling of the pulmonary artery (upper arrow) The coronary sinus is seen due to retrograde filling in both pictures

References

BLIX A S R HOL and O IVERSEN Equipment for Use in Angiographic Examinations of Animal during Diving *Acta physiol scand* 1973 81 3 A

ELSNER R W D L FRANKLIN and R L VAN CITTERS Cardiac Output during Diving in an Unrestrained Sea Lion *Nature* (Lond) 1964 202 809—810

FERRANTE F L and D F OPDYKE Mammalian Ventricular Function during Submersion Asphyxia *J appl Physiol* 1969 26 561—570

KJEKSHUS J J LEKVEN and O D MJOS Mechanisms in Maintenance of Cardiac Output during Coronary Insufficiency *Acta physiol scand* 1972 84 54 A

SCHOLANDER P F Experimental Investigations on the Respiratory Function in Diving Mammals and Birds *Hvalraadets Skrifter* Norske Vidensk-aps Akad Oslo 1940 22 1—131

Acta physiol scand 1973 87 433—447
From the Department of Physiology University of Goteborg Sweden

An Indicator Dilution Method for Studying Intestinal Hemodynamics in the Cat

By

BJORN BIBER OVE LUNDGREN LARS STAGE and JOAR SVANVIK

Received 21 June 1972

Abstract

BIBER B O LUNDGREN L STAGE and J SVANVIK *An indicator dilution method for studying intestinal hemodynamics in the cat* Acta physiol scand 1973 *87* 433—447

An indicator dilution technique is described for studying intestinal mucosal red cell and plasma flow and volume quantitatively and muscularis red cell and plasma flow and volume qualitatively The technique involves a close intra arterial injection of an intra vascular β radiating tracer and its detection with sensoring devices placed in the gut lumen and outside the intestine at the antimesenteric border Knowing the amount of tracer injected and total venous outflow it is possible to estimate regional flow from the height of the registered curve and regional blood volume from its area Since the volume of the region monitored by the detector depends on the energy level of the β radiation ^{32}P labelled red cells and plasma colloids used in this study were monitored from the whole mucosa while ^{198}Au labelled plasma particles were registered only from the villi This paper presents the theoretical background of the technique and some model experiments

---

In the recent years several techniques have been developed for the study of blood flow distribution within organs These techniques have made it possible to study separately for example blood flow in the renal cortex or in the gray matter of the brain and hence relate local flow to function The different methods make use of tracers which are either non diffusible (*e g* labelled red cells or serum albumin microspheres) or diffusible (*e g* heat inert gases water rubidium)

The blood flow distribution of the small intestine has been investigated with various accumulation techniques using microspheres (Grim and Lindseth 1958) rubidium (Csernay Wolf and Varro 1965) and DHO (Rayner MacLean and Grim 1960 Weiner 1961 Weiner and Grim 1966) However these methods only allow for one determination per animal while the inert gas washout technique allows repeated determinations This latter method was recently utilized for a detailed study of blood flow of the different intestinal wall layers (Lundgren 1967 Kampp and Lundgren 1968 Kampp Lundgren and Sjostrand 1968 a)

TABLE I

A	area under the recorded curve
$\bar{E}$	mean efficiency with which the tracer is detected when it is evenly distributed in the volume V
$\bar{E}_o$	mean efficiency with which the tracer is detected when it is evenly distributed in the volume surrounding the detector
$\bar{e}_i$	mean efficiency for a tracer particle during its transit in pathway i
$e_i(t)$	efficiency for a tracer particle passing in pathway i
F_{tot}	flow at the place of injection
F	flow per min and 100 ml of tissue volume
f_i	flow in pathway i
I	counting rate detected from tracer particles in all pathways
I_b	counting rate when the detector is immersed in blood
I_{max}	maximal counting rate recorded after an injection of tracer
I_{eq}	counting rate recorded at a constant concentration of tracer in the circulating blood and with the detector in situ
I_{inj}	counting rate when the detector is immersed in the injected tracer solution
i	any pathway for a tracer particle
$i_i(t)$	counting rate recorded from a tracer particle in pathway i
M_{tot}	amount of injected tracer
m	amount of tracer passing per 100 ml of tissue volume after an intraarterial injection
m_i	amount of tracer passing in pathway i
m_{inj}	amount of tracer per 100 ml of the injected tracer solution
V	volume per 100 ml of tissue available for tracer particles
V_{inj}	volume of the injectate
v_i	volume of pathway i
$\bar{t}$	mean transit time
t_i	transit time for blood and tracer particles in pathway i

The use of diffusible tracers is however in the intestine complicated by the existence of a countercurrent exchanger in the intestinal mucosa (Lundgren 1967) The i a injected tracer is short circuited extravascularly in this exchanger (Kampp Lundgren and Sjostrand 1968 b) and will consequently be partly excluded from the intestinal villi To circumvene these difficulties experiments have been performed in which regional blood flow in the small intestine is investigated by i a injected radioactively labelled red cells or plasma colloids which are not trapped in the mucosal countercurrent exchanger Their transit through the intestinal mucosa submucosa or muscularis is monitored by properly placed detectors A similar approach was recently used by Bacaner and Pollycove (1962) Bacaner and Beck (1964) and Bacaner (1966) but in these experiments the tracers were injected i v making a quantitative analysis almost impossible

In the present study the indicator dilution curves were recorded after *intra arterial* administration mucosal blood volume and blood flow being estimated from the dilution curves Thus the present method makes it possible to determine blood flow in the mucosa the functionally most important part of the small intestine After presentation of the theoretical background and the technique some pertinent methodological questions are discussed in connection with certain model and control experiments In subsequent papers the results obtained during resting blood flow during induced vasodilatation and during sympathetic nerve vasoconstriction will be presented

Preliminary reports of this study have been published elsewhere (Biber *et al* 1969 1971)

Methods

A Theoretical considerations
Wolgast (1968) outlined the theory of indicator dilution techniques as registered by an external detector above the tissue after an i a slug injection of an intravascular β emitting tracer In his paper Wolgast discusses various ways of determining mean transit time $\bar{t}$, which together with the regional blood volume V would give the flow F according to the formula

$$F = \frac{V}{\bar{t}} \quad (1)$$

An attempt is made below to discuss the theory of indicator dilution techniques from a somewhat different point of view although still largely based on Wolgast's reasoning The abbreviations used are presented in the text and in Table I

I Determination of regional blood flow At slug injection of an amount of tracer M_{tot} is made into a circulatory system with one inflow channel dividing into different parallel coupled circuits surrounding a detector in a symmetrical manner If the system flow expressed in ml/min is F_{tot} and the flow of a region is F ml/min per 100 ml tissue volume then the amount of tracer per 100 ml tissue volume m passing that region is

$$m = \frac{F}{F_{tot}} M_{tot} \quad (2)$$

During passage in pathway i the tracer is detected with a variable efficiency e (t) The counting rate i (t) from pathway i is *then*

$$i\ (t) = m_i\ e_i\ (t) \quad (3)$$

where m_i is the amount of tracer passing through pathway i It should be noted that e (t) is a function of time

The counting rate from all pathways in a region is

$$I = \sum_{i=1}^{i=n} m_i e\ (t) \quad (4)$$

If all the indicator passing the monitored region is assumed to be in the region and to be evenly distributed there at the time when I is maximal then

$$I_m = m\ \bar{E}_o \quad (5)$$

where $\bar{E}$ is the efficiency of the evenly distributed indicator

From equations (2) and (5) I_m can be expressed as

$$I_m = \frac{F}{F_t} M_{tot} \bar{E} \quad (6)$$

M_{tot} $\bar{E}_o$ can be quantitated if the detector is immersed in the injected tracer solution The counting rate then registered I_{tag} is the product of the amount of tracer m_t per 100 ml and the efficiency $\bar{E}_o$

$$I_{tag} = m_{tag}\ \bar{E}_o \quad (7)$$

However

$$M_{tot} = m_{tag} \frac{V_{inj}}{100} \quad (8)$$

where V_{inj} is the volume of injected tracer solution expressed in ml

Equations (6) (7) and (8) give

$$F = \frac{I_m}{I_{tag}} \frac{F_{tot}}{V_{inj}} 100 \quad (9)$$

The discussion above was carried out assuming that there existed a blood "particle" representative for the behaviour of blood in vessels. It is however well established that

pass faster through a tissue than plasma. It can be shown by an analogous discussion to the one above that if the tracer is bound to the red cells regional erythrocyte flow per 100 ml tissue equals

$$\frac{I_m \cdot F_{tot} \cdot Hct}{I_{tag} \cdot V_{tot}} \tag{9e}$$

where Hct denotes arterial hematocrit in per cent and if using labelled plasma particles that regional plasma flow per 100 ml tissue equals

$$\frac{I_m \cdot F_{tot}(100-Hct)}{I_{tag} \cdot V_{tot}} \tag{9p}$$

2. *Determination of regional blood volume.* The regional blood volume in the mucosa of the small intestine can be determined in two ways: a) from the area under the recorded indicator dilution curve (below called the "slug injection method") and b) from the activity recorded by the detector when the injected tracer has become equilibrated in the whole blood volume of the animal (below called "equilibration method").

a. Slug injection method. The fraction of the injected tracer which is passing the pathway i is supposed to equal the fraction of flow f_i/F_{tot} to this pathway

$$\frac{m_i}{M_{tot}} = \frac{f_i}{F_{tot}} \tag{10}$$

Thus equation (3) can be rewritten as

$$n_i(t) = \frac{M_{tot} \cdot f_i}{F_{tot}} \cdot e_i(t) \tag{11}$$

Integration with respect to time gives

$$\int_0^\infty n_i(t)\,dt = \frac{M_{tot}}{F_{tot}} f_i \int_0^\infty e_i(t)\,dt = \frac{M_{tot}}{F_{tot}} f_i t_i \frac{\int_0^\infty e_i(t)\,dt}{t_i} \tag{12}$$

where t_i is the transit time for the tracer and the blood particles in pathway i.

However $\frac{\int_0^\infty e_i(t)dt}{t_i}$ is the mean efficiency $\bar{e}_i$ for the tracer particle during its transit in the pathway i. Furthermore $f_i \cdot t_i = v_i$ where v_i is the volume of pathway i.
The equation (12) can therefore be written

$$\int_0^\infty n_i(t)\,dt = \frac{M_{tot}}{F_{tot}} v_i \cdot \bar{e}_i \tag{13}$$

The total number of counts registered from all pathways within the monitored volume which equals the area under the recorded curve A is then obtained as

$$\sum_{i=1}^{n} \int_0^\infty n_i(t)\,dt = A = \frac{M_{tot}}{F_{tot}} \sum_{i=1}^{n} v_i \cdot \bar{e}_i = \frac{M_{tot}}{F_{tot}} \cdot V \cdot \bar{E} \tag{14}$$

where V means the volume per 100 ml tissue available for tracer particles and $\bar{E}$ means the mean efficiency with which the detector records the tracer if it is evenly distributed into the volume V.

Equations (7), (8) and (14) give

$$A = \frac{I_{tag} \cdot V_{tot}}{F_{tot}} \cdot \frac{V}{100} \cdot \frac{\bar{E}}{\bar{E}_0} \tag{15}$$

If $\bar{E} = \bar{E}_0$, i.e. if the volume available for the indicator is evenly distributed in the monitored volume equation (15) can be written as

$$V = \frac{A \cdot F_{tot}}{I_{tag} \cdot V_{tot}} \cdot 100 \tag{16}$$

The discussion above refers to a hypothetical truly representative blood particle. However it can be demonstrated that if tagged red cells are used as the tracer the red cell volume per 100 ml equals

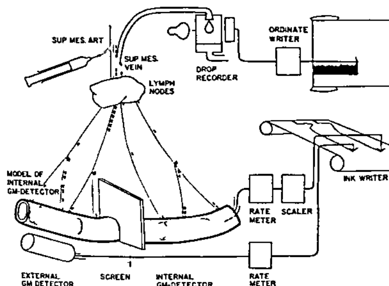


Fig 1 Schematic illustration of the technique used for studying the mucosal and muscularis haemodynamics in the small intestine of the cat *in situ* The venous outflow from the intestinal segment is continuously measured and small amounts of intravascular tracers are repeatedly injected in the intact superior mesenteric artery and their transit in the intestinal wall layers are monitored by properly placed β sensitive detectors

$$\frac{V\ F_t\ Hct}{I_{tag}\ V_{tj}} \tag{16r}$$

and if labelled plasma particles are used as the tracer the plasma volume per 100 ml equals

$$\frac{V\ F_t\ (100-Hct)}{I_{tag}\ V_{tj}} \tag{16p}$$

b Equilibration method If the tracer concentration in the circulating blood is kept constant V can be estimated by comparing the counting rate I_{reg} registered with the detector in situ symmetrically surrounded by the tissue and the counting rate I_b when the same detector is immersed in the circulating blood Then

$$V = \frac{I_{reg}}{I_b}\ 100 \tag{17}$$

if the efficiency factor is supposed to be the same If tagged erythrocytes are used as the tracer particles it can be shown that regional red cell volume per 100 ml equal

$$\frac{I_{reg}\ Hct}{I_b} \tag{17r}$$

and if labelled plasma particles are used regional plasma volume per 100 ml equals

$$\frac{I_{reg}\ (100-Hct)}{I_b} \tag{1 p}$$

B Operative procedures and determination of venous blood flow

The experiments were performed on cats anesthetized with chloralose (40—60 mg/kg b w) after ether induction The animals had been deprived of food at least 24 h and had no obvious signs of intestinal infection

The operative procedures used were similar to those of previous studies of intestinal circulation (*cf* Folkow Lundgren and Wallentin 1963 Kampp Lundgren and Sjostrand 1968 see also Fig 1) The abdomen was opened in the midline and the greater omentum and the spleen were extirpated The spleen was first electrically stimulated to expell its blood A segment of the

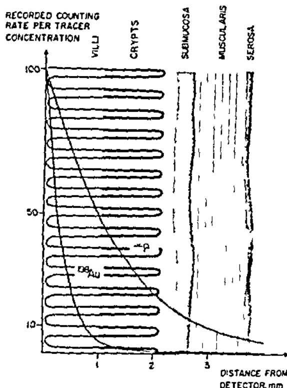


Fig 2 Schematic illustration of the steric efficiency pattern for ^{32}P and ^{198}Au with a well fitting cylindrical β sensitive detector in the intestinal lumen The ordinate represents the outer surface of the detector The counting rate per tracer concentration close to the detector has arbitrarily been set to 100 The average dimensions of the upper jejunum of a cat as estimated from frozen transverse intestinal sections from 10 cats are also included in the figure Note that the monitored ^{198}Au activity emanates from the villous tissues while the ^{32}P activity is registered from the entire mucosa

jejunum weighing 10—30 g was isolated and the remainder of the intestinal tract extirpated The lumen of the jejunal segment was flushed with bodywarm Tyrode solution or saline until the leaving fluid was clear The influence of the sympathico adrenal system was eliminated by bilateral sectioning of the splanchnic nerves and by denervation of the left adrenal gland and by exclusion of the right one from blood circulation by ligatures The vagal influence was eliminated by i v atropine 1 mg/kg b w

After heparinization the right femoral artery was connected to a mercury manometer or to a Statham pressure transducer to record mean arterial pressure The superior mesenteric vein draining all blood from the jejunal segment and its lymph nodes was cannulated and connected to an optical drop-recorder unit operating an ordinate writer (Fig 1) Venous outflow pressure could be set at any desired level by adjustments of the end of the tube draining the drop recorder but was usually kept around 10 mm Hg The venous blood was returned to the animal *via* a funnel and a catheter in the right jugular vein

To check the intravascular distribution of the injected slug one or two intestinal segments were cut open at the antimesenteric border in eight experiments and placed on a metal frame with an intact vascular supply as earlier described by Kampp Lundgren and Sjostrand (1968 b) In this way the mucosal surface covered by saline at a temperature of 38 C could be inspected for the appearance and distribution of i a injected slugs of plasma coloured by Evans blue (10 mg per ml plasma)

Bleeding was carefully avoided during the operative procedures and if occurring substitution by i v infusions of body warm Dextran Tyrode solution was performed Body temperature was maintained constant at 38 C by means of a thermostatically regulated heating pad

At the end of each experiment the weight of the intestinal preparation was determined for calculations of blood flow in ml/min×100 g. Furthermore in all experiments an adjacent jejunal segment was extirpated and frozen in a dry ice acetone mixture. A transverse section of this segment was photographed to check the intraluminal position of a detector model (Fig 3)

C Isotope techniques

1 Labelling of red cells The red cells were labelled with ^{32}P a pure β emitter (mean β energy 0 70 MeV maximum β energy 1 71 MeV) by mixing 8—10 ml of blood in a siliconized glass syringe with 1—2 ml of a citrate phosphate solution (Mollison *et al* 1958) containing per 100 ml water 3 0 g trisodium citrate sodium dihydrogen phosphate 0 015 g glucose 0 ? g The blood sample was then centrifugated at 135 g for 5 min and the plasma withdrawn Approximately 10 mCi of a ^{32}P phosphate solution (Na ^{2}-PO$_4$ AB Atomenergi Studsvik Sweden) was added to the red cells Pure oxygen was bubbled through this blood solution and the cells were incubated with the radioactive phosphate for 2 h at 30 C during gentle mixing The blood solution was then centrifugated and the cells washed with saline three times They were then resuspended in their own plasma During these procedures utmost care was taken to avoid hemolysis using well siliconized glassware

2 Labelling of plasma A solution containing colloidal $Cr^{32}PO_4$ (particle diameter ?00—400 Å or 1000—10 000 Å Sorin Italy) was used as a plasma marker in some experiments 2 mCi (0 5 ml) being mixed with 5—10 ml of blood In other experiments a colloid solution of ^{198}Au stabilized with gelatin (particle diameter 300 Å Farbwerke Hoechst AG) was used This tracer was injected into the superior mesenteric artery without previous mixing with plasma.

3 β radiation detectors When ^{32}P was used as tracer a cylindrical Geiger Muller tube (Philips nr 18509 sensitive length 17 5 mm diameter 5 0 mm dead time 27 μs) was inserted into the intestinal lumen (Fig 1 internal detector) and coupled to a linear ratemeter (Packard model 306) and a scaler (Packard Auto Gamma Series 410 A) The ratemeter (time constant 0 1 or 0 3 s) operated one channel of a Goertz RE 520 inkwriter

Another Geiger Muller tube (Philips nr 18550 length 28 mm diameter 8 mm dead time 36 μs) was in some experiments placed closely to the external intestinal wall at the antimesenteric border (Fig 1 external detector) opposite to an internal detector model distending the intestine An aluminium sheet connected to ground was placed between the internal and external detectors to eliminate their electrical interference The external G-M tube was coupled to a linear ratemeter (Packard Series 280 A) operating the other channel of the inkwriter

When using ^{198}Au (mean β energy 0 33 MeV maximum β energy 0 96 MeV) as the tracer a semiconductor specially designed by AB Atomenergi Studsvik, Sweden (AE 401) was used as internal detector (sensitive length 4 0 mm diameter 5 0 mm) Care was taken to keep the temperature of the detector constant since the observed counting rate was highly dependent of the temperature It was therefore measured inside the segment by means of a thermocouple thermometer (Electrolab Copenhagen) The semiconductor was coupled to a pulse height analyser discriminating in such a way as to almost exclude the γ radiation from ^{198}Au Model experiments (see below) showed that less than 5 per cent of the total recorded activity was γ radiation with the discriminaton setting used The pulse height analyser was coupled to a linear ratemeter and scaler (Packard model 306 and A to Gamma Series 410 A) and also to a tape perforater which gave ? punch outs per second The tape was read by a tape reader connected to a Hewlett Packard calculator (mod I 9100 A)

D Model experiments

To determine the geometric efficiency pattern of the internal detectors model experiments were performed with the detector submerged in Tyrode solution at 37 C ? o ^{198}Au were adhered by heat to a glass needle kept parallel to the detector at predetermined distances to this The needle was first placed in contact with the detector and then moved stepwise 0 2 mm counts being taken in each position It was tested that the tracer had not dissolved in the solution by determining background activity before and after the experiment

E Experimental procedures and calculations

0 05—0 10 ml of the ^{32}P labeled red cells or plasma or 0 01?5—0 03 5 ml of the ^{198}Au labelled colloid solution was injected within 0 1—0 ? s into the superior mesenteric artery (Fig 1) Most of the resulting indicator dilution curves were analysed manually with semilogarithmic extrapolation of the descending slope and the curve area was determined with a planimeter The curves obtained with ^{198}Au labelled plasma colloids were however transferred to perforated tape and fed into a Hewlett Packard calculator which extrapolated the descending slope using the method of least squares and calculating the curve a

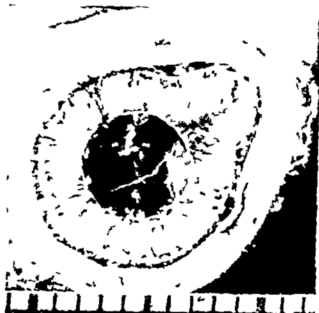


Fig 3 A frozen transverse section of a cat jejunum with an intraluminal bees wax model of a detector tube The intestinal segment was extirpated during a close intra arterial infusion of Evans blue coloured plasma Scale in lower part of figure denotes mm Note the close contact between tissue and detector surface

The equations of section A were used to determine regional erythrocyte (plasma) flow and erythrocyte (plasma) volume (slug injection method) A and $I_{m x}$ being determined from the indicator dilution curves $I_{t g}$ was obtained by diluting the injected volume with a known volume of Tyrode solution and the counting rate was registered by the submerged detector j was known and F_{tot} was continuously monitored (Fig 1) Arterial hematocrit was determined in heparinized capillary tubes centrifugated at 5000 rpm for 5 min

Mean transit time ($\bar{t}$ v (i)) was calculated from the indicator dilution curve by dividing the curve area (A) by the curve height (I_{max}) (Zierler 1965 Wolgast 1968)

For determining regional erythrocyte volume according to the equilibration method 1 2 ml of the labelled cells was iv injected while clamping the intestinal artery and vein for 3 min to avoid that free ^{32}P (i e not contained within the red cells) was trapped in the monitored time 10—15 min after the tracer administration when the internal detector signalled a constant rate an arterial blood sample (1 ml) was withdrawn This sample was diluted with 10 ml Tyrode solution in a beaker with 12 mm inner diameter into which the internal detector was submerged for registration of the counting rate The counting rate of the original blood was obtained by extrapolating back to the original volume

Results

A The monitored tissue volumes of ^{32}P and ^{198}Au

In model experiments (see Methods D) the steric efficiency pattern was determined for ^{32}P and ^{198}Au using a G M tube and a semiconductor detector respectively The counting rate recorded when a radiation source was placed at predetermined distances from the detector was multiplied by 2 π r r being the distance between the radiation source and the central axis of the detector Correction was thus made for the different volumes of the rotation bodies around the detector (see Wolgast 1968)

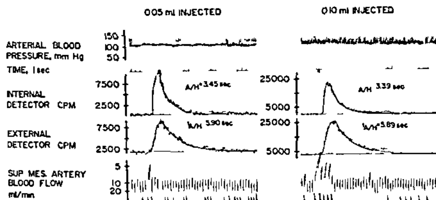


Fig 4 The indicator dilution curves registered by an internal and an external detector after slug injections (signal) of ^{32}P labelled red cells into the superior mesenteric artery during rest Injected volumes are indicated above each panel Arterial blood pressure and superior mesenteric artery blood flow were continuously measured as shown in upper and lower registrations respectively Note that the maximal height of the curves are doubled and that A/H is constant when the injected volume is increased from 0 05 to 0 10 ml

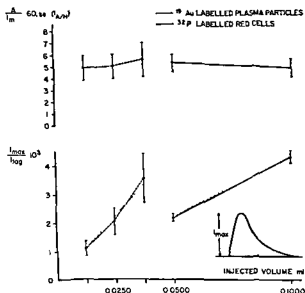


Fig 5 *Upper curve* Cumulated data on the relationship between injected volume of tracer and ratio between area (A) and maximal height (I_m) of the indicator dilution curve as recorded with an internal detector ^{198}Au particles were injected in 4 animals and ^{32}P labelled red cells in 8 expts In each expt. total intestinal blood flow was the same at the different injections Note the fairly constant A/I_m ratio despite different injected volumes

Lower curves Cumulated data on the relationship between injected volume of tracer and I_m The latter is here expressed in relation to the tracer concentration (I_{tag}) of the injected solution Data from the same expts as in upper curves Thin dotted lines indicate l n proportionality

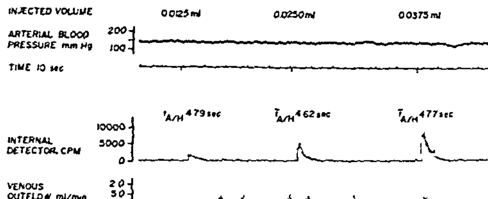


Fig. 6 The indicator-dilution curves registered by an intraluminal detector after slug injections (signal) of different volumes of colloidal ^{198}Au particles into the superior mesenteric artery during "rest" Arterial blood pressure and venous outflow from the intestinal segment were continuously recorded as depicted in upper and lower registrations respectively Note the proportional increase of the maximal height of the curves and the fairly constant $\bar{t}_{A/H}$ when the injected tracer volume is increased

From measurements made at 0.2 mm intervals a smoothed curve was computed as shown in Fig 2 in which the counting rate per tracer concentration has arbitrarily been set to 100 close to the detector The dimension of a "normal" cat small intestine is also shown in this Fig Clearly the internal detector records radioactivity from ^{198}Au only in the villi but from ^{32}P in the entire mucosa 50 (80) per cent of its activity being localized within 0.9 (2.0) mm from the detector at a homogeneous tracer distribution

A bees wax model of the detector was placed in an adjacent intestinal segment which was then frozen and photographed (see Methods section B) Fig 3 illustrates a representative experiment A good contact between the detector and the intestinal mucosa is seen although minor gaps occur To correct for this a computer program was developed based on the "efficiency" curves of Fig 2 and the cross section photographs of each experiment It could be calculated that the observed counting rate should be corrected for a loss of 6.4 ± 0.95 per cent (mean $\pm$ S.E. $n = 35$) using ^{32}P and 14.1 ± 1.88 per cent ($n = 35$) using ^{198}Au

Furthermore there is always a small space between the mucosal villi Considering these as cylinders ($r = 75$ μm) with spherical tips and as tightly packed as is theoretically possible a correction factor was also here calculated amounting to 7.3 per cent for ^{32}P and 15.4 per cent for ^{198}Au

B The indicator dilution curves

Fig. 4 illustrates an experiment during "resting" blood flow where ^{32}P labelled red cells were i.a. injected. The volumes injected are indicated above each panel Blood flow was here registered by a drop counter in the superior mesenteric artery and

radioactivity by one internal and one external G M tube The thin lines along the downward slope of the indicator dilution curves indicate the calculated exponential wash out showing their very good agreement with the registered ones

Fig 4 also illustrates the effects of doubling the injection volume on mean transit time ($\bar{t}_{\lambda\,II}$) and arterial blood flow Although flow registration is transiently decreased by the larger volume, calculated mean transit time is not altered This is also illustrated in Fig 5 summarizing 8 control expts The effects of the injected volumes on blood flow through the intestinal tissues are probably overestimated with the arterial flow registration of Fig 4 since the i a injections were made between the drop chamber and the intestine

Fig 6 illustrates in a similar manner the effects of changing the injected volume of ^{198}Au labelled plasma on mean transit time and venous outflow during rest Tripling the injected volume does not change either blood flow or calculated mean transit time This is also illustrated in Fig 5 summarizing 4 control expts Fig 5 also demonstrates for both tracers the linear relationship between the ratio I_m /$I_{t\,g}$ and the injected volume of tracer solution Thin dotted lines indicate line of proportionality

In 8 expts 0 025—0 050 ml plasma coloured with Evans blue was injected i a and the distribution of the slug in the intestinal mucosal vessels was followed by direct inspection of the mucosa (see Methods section B) An uneven colouring of the mucosa was sometimes seen particularly during resting conditions The heterogeneous distribution of the colour may be caused by myogenic activity in the precapillary sphincters and in order to relax these structures isopropylnoradrenaline was infused i a in some experiments Perfusion pressure and hence flow rate was controlled by a clamp around the superior mesenteric artery In these experiments the mucosa became homogeneously coloured The distribution of the injected slug was further tested by inspecting simultaneously one duodenal and one ileal segment Slugs injected at different flow rates seemed to be evenly and equally distributed to the surface of the two segments

C Determination of regional blood flow and blood volume

To exemplify how regional blood flow and blood volume is measured the calculations based on the middle curve of Fig 6 will be given in detail The computer reading off the perforated tape of this curve determined the exponential equation of its descending slope and could then calculate the curve area (A) to 223 counts The maximal height (I H) of the curve amounted to 2900 counts/min these data allow an estimation of mean transit time ($\bar{t}_{\lambda\,II}$ eq 1) to 4 62 s

Regional plasma flow was estimated from eq 9_p in Methods Since loss of radioactivity due to geometrical factors (see Results A) amounted to 23 6 per cent $I_{m\,x}$ was multiplied by 1 30^c $\left(= \frac{100}{100\ 23\ 6}\right)$ giving a corrected $I_{m\,x}$ of 3796 cpm $F_{t\,t}$ was 4 3 ml/min as continuously recorded by the drop counter The injected volume

was 0 025 ml keeping a tracer concentration giving 2,749 000 counts/min ($I_{t,g}$) Arterial Hct was 31 per cent From these values a regional plasma flow in the monitored tissue of 16 4 ml/min × 100 ml tissue was calculated

Regional plasma content was estimated from eq 16p in Methods After correction the curve area amounted to 291 counts The other values identical to those above, were giving a regional plasma volume of 1 26 ml/100 ml tissue

Discussion

The theory of indicator dilution methods is based on certain assumptions implying that the tracer is instantaneously injected and thoroughly mixed with blood and hence that tracer flow is really representative for total flow It is furthermore assumed that the intravascular flow and volume remains unchanged while the indicator dilution curve is recorded (see e g Zierler 1962) The tracer injection of the present study lasted only 0 1—0 2 s i e they were almost instantaneous in relation to the recorded mean transit time which was never shorter than 0 8 s even at maximal vasodilatation The presence of complete mixing between tracer and blood is difficult to assess experimentally but the linear relationship between injected volume and I /$I_{t,g}$ (Fig 4 5 and 6) suggests that mixing is acceptable otherwise a non linear relationship would have been observed Further this conclusion is also suggested by the findings in the experiments using Evan s blue coloured plasma

A constant venous outflow during an injection was taken to indicate that the intestinal blood flow pattern remained unchanged Injections causing large changes of total blood flow were discarded Furthermore the reduction of hematocrit by injecting only 0 0125—0 0375 ml of a ^{198}Au plasma solution was regarded to be negligable the ^{32}P labelled red cells and plasma particles were injected mixed in blood

The theoretical background for using external detectors to record indicator dilution curves from a tissue surface was outlined by Zierler (1965) and Wolgast (1968) Zierler developed the theory for mean transit time and regional flow calculations using an external detector after an arterial slug injection of a radioactive tracer which was registered with more or less equal efficiency from the whole monitored region Wolgast further developed these concepts for conditions where the monitored volume was limited and defined by the maximal radiation energy of the tracer Thus the activity within the monitored tissue volume was among other things a function of the distance to the detector

From the discussions of these authors one may conclude that there exists no completely accurate method for determination of mean transit time with external detectors for slug injections of tracers However mean transit time calculated by dividing the curve area (A) with its maximal height (I_{max}) will give an accurate value in a tissue where according to Wolgast (1968) ' the transit times in afferent vessels to the different capillary regions would be equal or short compared to the transit times in the capillaries Further the efficiency factor must on the average be smaller for

the indicator during the transit through the afferent vessels than when the indicator is in the capillary system

Such anatomical prerequisites, seem to be fulfilled in the intestinal mucosa which was of primary interest in the present study Thus arterial supply to each villus consist of a single tortuous vessel running in its central part without branching but arborizing at the tip into a dense subepithelial capillary network In the cat the capillaries collect into veins at the villous base (Heller 1872 Nisioka 1927) In such a vascular network the linear flow rate must be several times faster in the central artery than in the capillaries The tracer is therefore quickly brought to the very dense capillary network where its transit is slow before it is transported away *via* the veins at the villous base

Apart from the anatomical assumptions the theoretical considerations outlined in Methods (section A) imply that the tracer passing the monitored region is here evenly distributed within the monitored volume at the time of maximal counting rate (I_{max}) (Wolgast 1968) This crucial assumption means that 1) the intra vascular volume must be evenly distributed in the monitored tissue volume 2) the tracer must be evenly distributed in this intravascular volume at I_{max} 3) the entire tracer amount passing the monitored tissue volume must be located there during I_{max}

Ad 1 The villous intravascular volume is probably fairly homogeneously distributed to judge by the even colouring upon i a injection of *e g* Evans blue (Fig 3) Just below the villi a more bleached region is observed suggesting a less vascularized region but a well vascularized region seems to exist at the crypt bases (Lundgren 1967) This latter conclusion is also corroborated by Fig 3 Finally Jodal and Lundgren (1970 b) showed that the intestinal submucosa per unit weight contains 5—7 times more blood than the mucosa and muscularis Taking the steric efficiency curves of Fig 2 into consideration these observations indicate that the monitored intravascular volume is evenly distributed when ^{198}Au is used as tracer With ^{3}P labelled red cells or plasma colloids on the other hand the monitored blood volume is not evenly distributed although the submucosal tracer is recorded with low efficiency (Fig 2)

To obtain a correct value for blood content per unit tissue volume correction must be made for the fact that the volume monitored by the internal detector is partly comprised of the intervillous space and some secretion between detector and mucosa A correction for this was made possible by means of a computer program based on the efficiency curves of Fig 2 and the photographs of the detector model *in situ* (Fig 3)

Ad 2 It was proposed by Jodal and Lundgren (1970 a) that the dynamic hematocrit in the intestinal mucosa is considerably lower than arterial hematocrit partly due to a considerable plasma skimming mechanism probably occurring in the submucosa and/or in deeper mucosal parts Hence red cells and plasma are not uniformly distributed when the monitored volume includes these parts of the intestinal wall as when ^{3}P is used (*cf* Fig 2) but with ^{198}Au the hematocrit of the

monitored blood volume is probably fairly constant

Ad 3 This cannot *a priori* be considered to be true but the experiments shown in Fig 4 5 and 6 demonstrate that measured mean transit times expressed as $\bar{t}_{XII}$ were similar when injecting different tracer volumes This possibly indicates that these injected volumes were small as compared to the detected volume

In conclusion it may be stated that the theoretical assumptions inherent in the indicator dilution technique seem to be adequately fulfilled as regards the use of the internal detector although no quantitative evaluation of the methodological error is possible An external detector was utilized when ^{32}P labelled red cells were used The mean efficiency of the detection could not be determined and hence I_{tag} of equation 9 and 16 could then not be estimated Consequently *absolute* values for regional flows and volumes could not be calculated but *relative* changes were detectable Mean transit time ($\bar{t}_{XII}$) could however be determined since E need not to be known in such calculations

This research has been sponsored by grants from the Swedish Medical Research Council (No B 72 14X 2855 03 and partly by B 72 14X 16-08 A) from the Faculty of Medicine University of Goteborg and from Svenska Sallskapet for Medicinsk Forskning

AB Vitrum generously supplied us with heparin Thanks are also due to AB Pharmacia for its supply of Rheomacrodex® and Macrodex®

References

BACANER M B Quantitative measurement of regional colon blood flow in the normal and pathological human bowel *Gastroenterology* 1966 *51* 764—777

BACANER M B and J S BECK Regional blood flow measurement in vivo for a definable geometry *Amer J Physiol* 1964 *206* 962—966

BACANER M B and M POLLYCOVE Determination in vivo of regional circulatory dynamics in intact intestine *Amer J Physiol* 1962 *203* 1094—1102

BIBER B O LUNDGREN and J SVANVIK An indicator-dilution technique for studying mean transit time and blood flow in the mucosa submucosa of the small intestine *Acta physiol scand* 1969 Suppl 330

BIBER B O LUNDGREN and J SVANVIK An indicator dilution technique for studying erythrocyte and plasma flows and volumes in the intestinal mucosa submucosa of the cat Abstract XXV *Int Congr Physiol Sciences* 1971 Munich

CSERNAY L F WOLF and V VARRO Der Kreislaufgradient im Dunndarm *Z Gastroenterologie* 1965 *3* 261—265

FOLKOW B O LUNDGREN and I WALLENTIN Studies on the relationship between flow resistance capillary filtration coefficient and regional blood volume in the intestine of the cat *Acta physiol scand* 1963 *57* 270—283

GRIM E and E O LINDSETH Distribution of blood flow to the tissues of the small intestine of the dog *Univ Minn med Bull* 1958 *30* 138—145

HELLER A Über die Blutgefasse des Dunndarmes *Ber sachs Ges Wiss* 1872 *24* 165—171

JODAL M and O LUNDGREN Plasma skimming in the intestinal tract *Acta physiol scand* 1970 a *80* 50—60

JODAL M and O LUNDGREN Regional distribution of red cells plasma and blood volume in the intestinal wall of the cat *Acta physiol scand* 1970 b *80* 533—537

KAMPP M and O LUNDGREN Blood flow and flow distribution in the small intestine of the cat as analysed by the Kr⁸⁵ wash out technique *Acta physiol scand* 1968 *72* 282—297

KAMPP M O LUNDGREN and J SJOSTRAND On the components of the Kr⁸⁵ wash-out curves from the small intestine of the cat *Acta physiol scand* 1968 a *72* 257—281

KAMPP M O LUNDGREN and J SJOSTRAND The distribution of intravascularly administered lipid soluble substances in the mucosa and the submucosa of the small intestine of the cat *Acta physiol scand* 1968 b *72* 469—480

LAUBER A and M WOLGAST Needle Type solid state detectors for in vivo measurement of tracer activity *J Physics in medicine and biology* In press

LUNDGREN O Studies on blood flow distribution and countercurrent exchange in the small intestine of the cat. *Acta physiol scand* 1967 Suppl 303
MOLLISON P L, M A ROBINSON and D A HUNTER Improved method of labelling red cells with radioactive phosphorus *Lancet* 1958 *12* 766—769
NISIOKA T 1927 Cited by Spanner 1932
RAYNER R L MACLEAN and E GRIM Intestinal tissue blood flow in shock due to endotoxin *Circulat Res* 1960 *8* 1212—1217
SPANNER, R. Neue Befunde uber die Blutwege der Darmwand und ihre funktionelle Bedeutung *Morph Jb* 1932 *69* 394—454
WEINER D *Kinetics of distribution of D_2O in the tissues of the canine ileum* (Thesis) Minneapolis Univ of Minnesota 1961 Quoted from Grim E The flow of blood in the mesenteric vein *Handbook of Physiology* Sect 2 Vol II 1963 1439—1456
WEINER D E. and E GRIM Kinetics of distribution of D_2O in canine intestinal tissues *Amer J Physiol* 1966 *211* 600—606
WOLGAST M Studies on the regional renal blood flow with I^{3} labelled red cells and small beta sensitive semiconductor detectors *Acta physiol scand* 1968 Suppl 313
ZIERLER K. L. Theoretical basis of indicator dilution methods for measuring flow and volume *Circulat Res* 1962 *10* 393—407
ZIERLER K L Equations for measuring blood flow by external monitoring of radioisotopes *Circulat Res* 1965 *16* 309—321

Acta physiol scand 1973 87 448—454

From the Institute of Physiology University of Oslo Norway

A Two-Phased Change in Dynamic Lung Compliance during Hemorrhagic Hypotension

By

GUNNAR BO and ANTON HAUGE

Received 28 June 1972

Abstract

BO G and A HAUGE *A two phased change in dynamic lung compliance during hemorrhagic hypotension* Acta physiol scand 1973 87 448—454

Prolonged hemorrhagic hypotension and multiple trauma not involving the lungs can in patients induce respiratory failure with a fall in lung compliance and a concomitant increase in the work of breathing (Moore *et al* 1969) However animals experiments have revealed conflicting results concerning the magnitude and direction of change in lung compliance after a hemorrhage We have studied the effects of 3 hr of hypovolemic systemic hypotension (femoral a pressure 50 to 60 mm Hg) on lung mechanics in open chest cats ventilated by positive pressure

Mean dynamic lung compliance (C_L) rose during the first 30 min post bleeding by 13 ± 5 per cent whereafter C_L gradually fell to 26 ± 6 per cent below its initial value in the third hr of observation On the basis of our monitoring of vascular parameters and alveolar arterial P_{O_2} differences we suggest that the early rise in C_L is due to reduced pulmonary blood volume and vascular distending pressure whereas the later fall in C_L is caused by collapse and narrowing of peripheral airways No significant changes in nonelastic (frictional) resistance could be detected during the 3 hr observation period

Hemorrhagic systemic hypotension and multiple trauma not involving the lungs may cause life threatening respiratory failure in patients (Moore *et al* 1969) The cause and mechanism of this deterioration of the lung function is almost unknown *Stiffening of the lungs or reduction of lung compliance* with an increase in respiratory work as an essential consequence is a prominent feature of this lung syndrome (Moore *et al* 1969)

The effects of hemorrhagic systemic hypotension upon lung compliance seem however to be controversial Most investigators have reported a decline in lung compliance during hemorrhage (Gerst *et al* 1959 Salzano and Hall 1961, Henry *et al* 1967) On the other hand significant increases in lung compliance have also been observed (Cahill and Byrne 1964 Proctor *et al* 1969) The conflicting results may to some extent be due to different treatment of the animals *e g* in the degree and duration of systemic arterial hypotension or in the type of ventilation procedures used

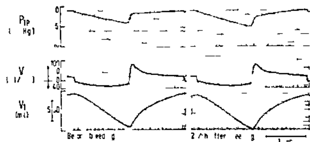


Fig. 1. Transpulmonary pressure (P_{TP}), tracheal airflow ($\dot{V}$) and ventilation volume (V_T) recorded during intermittent positive pressure ventilation. The animal is ventilated with constant pump stroke volume and a frequency of 21/min. The left panel demonstrates the situation before withdrawal of blood, the right panel demonstrates the situation after 3 h of systemic arterial hypotension and hypovolemia.

The purpose of the present work was to investigate the effect of hemorrhagic hypotension upon lung compliance by using a pneumo-tachygraph technique which permits repeated measurements in the same animal. Furthermore we wanted to elucidate the mechanism behind the observed changes in lung compliance by simultaneously monitoring of relevant vascular parameters.

Methods

Anesthesia and circulatory parameters. Cats were anesthetized with i.p. injections (30 to 40 mg/kg) of sodium pentobarbitone (Nembutal® Abbott). Thorax was opened by a sternum splitting incision. Polyethylene catheters were introduced into the femoral artery, the pulmonary artery and the left atrium for recordings of the femoral arterial pressure (P_{FA} with a Statham P23Gb transducer), the pulmonary arterial pressure (P_{PA} with a Statham P23Db transducer) and the left atrial pressure (P_{LA} with a Statham P23De transducer). Cardiac output was measured by the use of a wraparound flow transducer on the ascending aorta connected to a Nycot on square wave electromagnetic flow meter (type 3/2 Nycotron A/S, Norway).

Ventilation. The animals were ventilated with room air through a tracheal cannula using a positive pressure pump (The Ideal Respiration Pump, C. F. Palmer Ltd., London). The ventilation frequency was kept at 21 strokes per min in all the experiments. The stroke volume was adjusted to obtain a normal arterial pH until the start of bleeding, whereafter it was kept constant throughout the experimental period. The end expiratory tracheal pressure was maintained at a positive value of 1 cm H_2O.

Lung compliance. Between the ventilation pump and the tracheal cannula a screen pneumotachygraph designed for small animals was inserted (Hewlett Packard). The pressure difference across the screen was calibrated to air flow and measured by a differential pressure transducer (model 270, Hewlett Packard). The air flow signal was integrated electronically (Respiratory Preamplifier model 350—5000 B, Hewlett Packard) and recorded as tidal volume. The tracheal pressure which under open chest conditions is equal to transpulmonary pressure was measured by a pressure transducer (model 270, Hewlett Packard). Tracheal airflow and pressure and the tidal volume were continuously recorded during the experimental period on a Sanborn recorder (Model 320, Hewlett Packard). Every half hour these parameters were recorded with a graph paper speed of 5 cm/s (Fig. 1). Lung compliance was calculated as the ratio between the tidal volume and the transpulmonary pressure difference between start and end of a tidal volume change. The tracheal pressures used to obtain this pressure difference were read at the end tidal positions with zero tracheal air flow (Fig. 1). Lung compliance measured in this way is conventionally called dynamic lung compliance.

Every half hour the expiratory tube was clamped until the peak inspiratory pressure reached about 15 cm H_2O. All compliance measurements were carried out 10 min after such a deep lung inflation except when otherwise stated.

Non elastic (frictional) resistance was calculated by taking the difference in tracheal pressure at equal lung volumes within one respiratory cycle and dividing this pressure difference by the sum of the flow rates at these two points. Using this method we assumed that the flow at the points measured is on the linear part of the nonelastic pressure flow curve and furthermore that the resistance during inspiration is the same as during expiration. The resistance of the tracheal cannula was not subtracted.

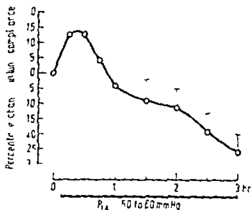


Fig 2 Percentage change in lung compliance during 3 h of systemic arterial hypotension. Mean values from 8 animals are given The vertical line indicate 2 times standard error of the mean P_{FA} = femoral arterial blood pressure

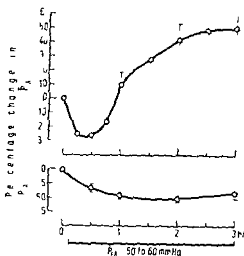


Fig 3 Percentage change in pulmonary arterial pressure (P_{PA}) and left atrial pressure (P_{LA}) during 3 h of systemic arterial hypotension Mean values from the same 8 animals as represented in Fig 2 are given. The vertical lines indicate 2 times standard error of the mean

Arterial oxygen tension was measured by a Radiometer Po electrode (type E 5046) connected to an Acid Base Analyzer (type PHM 71) equipped with a Po module For pH measurements in arterial blood a Radiometer pH meter 22 was used

Standardized hemorrhagic hypotension was induced by bleeding the animal through the femoral artery into a graded syringe until mean femoral arterial pressure had fallen to a level between 50 and 60 mm Hg Femoral arterial pressure was maintained at this level by appropriate small extra withdrawals or infusions of blood

Results

We found the initial mean value for dynamic lung compliance in 16 cats to be 2 66 ml/cm H O per kg Eight of these cats were bled until mean systemic blood pressure had fallen to between 50 and 60 mm Hg The animals were then observed for a period of 3 h The immediate effect of the withdrawal of blood was a rise in lung compliance by 13 ± 5 per cent (Mean ± S E) (Fig 2) After 30 to 60 min a gradual fall in compliance took place to a mean level of 26 per cent (S E ± 6) below initial value In 6 of these bled animals the shed blood was retransfused after

TABLE I The development of dynamic lung compliance in per cent of initial value in the control group of animals

Time in hours	0	½	1	1½	2	2½	3
Dynamic lung compliance in per cent of initial (zero time) value ± S.E.	100	102 ±7.8	97 ±6.7	97 ±5.6	100 ±6.7	100 ±13.0	93 ±4.4

TABLE II The development of lung nonelastic resistance (cm H_2O/l/s ± S.E.) in the control group of animals and in the bled animals

Time in hours	0	1	2	3
Non bled animals (n = 4)	24 ±6	22 ±4	25 ±5	27 ±8
Bled animals (n = 8)	22 ±5	21 ±6	18 ±3	21 ±8

the end of the 3 h period. Compliance was however not significantly changed by this procedure. After retransfusion at this late stage compliance was still found to be 28 ± 6 per cent (Mean ± S.E.) below initial value.

The changes in circulatory parameters were in agreement with previous observations on this animal model (Bø and Hognestad 1971). There occurred an early fall in pulmonary arterial pressure followed by a later rise with the same time course as the change in lung compliance (Fig. 2 and 3). By plotting the changes in compliance against the changes in pulmonary arterial pressure paired values revealed a significant inverse correlation ($r = -0.75$, $p = 1$ %) between these 2 variables.

The initial fall in pulmonary arterial pressure was caused by a reduction in cardiac output whereas the later rise in pressure reflected an increase in pulmonary vascular resistance (Bø *et al.* 1972 a). The cardiac output remained low in the range of 40 to 60 per cent of its initial value. Retransfusion of blood brought cardiac output up to a level slightly below its initial value and increased pulmonary arterial pressure further to a mean level of 66 per cent above initial value.

4 animals were treated in the same way as this experimental group with the only exception that no blood was withdrawn or transfused. Development of lung compliance in these control experiments is shown in Table I.

In order to examine more closely the effects on lung compliance of acute changes in blood volume 2 animals were bled rapidly (3 min) until the systemic arterial blood pressure had fallen to 50 mm Hg. Their shed blood was then rapidly (8 min) transfused into 2 normovolemic animals. Lung compliance was determined every minute. The results are demonstrated in Fig. 4. In the rapidly bled animals compliance increased by about 20 per cent and in the rapidly transfused animals

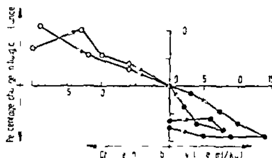


Fig 1 Effects on lung compliance of rapid withdrawals and transfusions of blood The open circles represent withdrawals of blood over periods of 3 min in 2 different animals The closed circles represent intravenous transfusions of blood over periods of 8 min into two other animals, and the subsequent withdrawal of the same blood volumes Deep lung inflations were not carried out during these studies.

compliance fell by up to 19 per cent of initial value When the extra blood volume was withdrawn from the two latter animals compliance did not return to initial value until one deep lung inflation had been carried out

A possible explanation for the observed fall in lung compliance during prolonged systemic hypotension might be closure of terminal airways For the evaluation of this hypothesis arterial oxygen tension (Pa_{O_2}) was determined after 10 min ventilation *with oxygen at ambient pressure* before bleeding and after 3 h of systemic hypotension We found Pa_{O_2} (S E ± 15) and 465 (S E ± 105) mm Hg respectively The latter value suggests a relative shunt flow through the lungs in the order of 10 per cent (Moore *et al* 1969 p 137)

Pa_{O_2} during ventilation with room air in the third hour of systemic hypotension was on the average 90 mm Hg (S E ±4)

Initial mean value for nonelastic resistance in all the animals was 22 5±5 cm H_2O/1/s (Mean±S E) In 5 of the 8 animals in the bled group there occurred a moderate fall in nonelastic resistance in 2 animals there was no change and in 1 animal a rise most likely due to excessive bronchial mucous secretion On the average *no significant change in nonelastic resistance occurred during the 3 h observation* period The results both from the non bled control group of animals and from the bled and hypotensive group are given in Table II

Discussion

When lung compliance is studied experimentally in animals the effects of time, anesthesia and artificial ventilation have to be taken into consideration Mead and Collier (1959) found a 37 per cent reduction in lung compliance after 2 h of anesthesia and steady mechanical ventilation in the dog Deep lung inflations repeated at intervals completely abolished such a fall in compliance In the present experiments we applied standardized hyperinflations every half hour and measured compliance at a fixed interval after the previous hyperinflation With this schedule we found a constant or near constant lung compliance during anesthesia and mechanical ventilation of the non bled control animals for at least 3 h indicating that the open thorax model and the type of ventilation chosen were adequate for the experimental purpose Moreover the mean pre bleeding and control value of

lung compliance of 2 66 ml/cm H_2O per kg is in accordance with previous reports (Spells 1969/70) Consequently, the two phased change in lung compliance found in the hypotensive animals must be due to effects of the hemorrhage and the ensuing hypovolemic state

It appears that previous workers have recorded one phase only of the change in lung compliance after bleeding By excluding the regular deep lung inflations Salzano and Hall (1961) detected the fall in compliance only When on the other hand the compliance measurements were done immediately after a deep lung inflation the second phase with a reduction in compliance seemed to be lost (Cahill and Byrne 1964)

The early and rapid rise in compliance found in the present experiments most likely reflects changes in pulmonary vascular pressures and pulmonary blood volume These are factors which are known to influence lung mechanics (Hughes *et al* 1958) In a previous work on similarly treated and bled animals Aarseth and Bo (1972) examined lung blood volume and lung extravascular water content 30 min and 3 h after bleeding at moments when the pulmonary arterial pressure is decreased respectively increased (Fig 3) After 30 min lung blood volume was reduced by 39 per cent and the lung water volume increased by 6 per cent The total vascular changes in the lung at this time were thus a markedly reduced blood volume and reduced pulmonary arterial and left atrial pressures Under otherwise identical conditions with respect to transpulmonary pressure and bronchial smooth muscle tone such changes in vascular parameters should give more space within the lungs for the expansion of airways Consequently a rise in lung compliance was to be expected The finding of a fall in lung compliance during rapid i v infusion of blood supports this explanation

The situation after 3 h of hypotension was in several ways different Lung blood volume at this stage is reportedly reduced by 20 per cent on the average and lung extravascular water volume is unchanged compared with control values (Aarseth and Bø 1972) We found left atrial pressure to be slightly below control value whereas pulmonary arterial pressure was increased by about 50 per cent (Fig 3) A critical question to answer is whether pulmonary arterial hypertension reduces lung compliance As demonstrated by Hughes *et al* (1958) and recently confirmed in our laboratory (Bo *et al* 1972 b) a 50 per cent increase in pulmonary arterial pressure obtained by a rise in flow reduced lung compliance by some 5 per cent only One would therefore expect the combined effects of the pulmonary vascular changes on lung compliance at this later stage to be negligible As an alternative explanation we suggest that the second phase with a fall in lung compliance is due to narrowing and collapse of peripheral airways

It has previously been found that the increase in pulmonary arterial pressure during prolonged hemorrhagic hypotension is caused partly by active vasoconstriction (Bo *et al* 1972 a) and partly by mechanical obstruction of lung vessels most likely by aggregates of blood platelets (Bo and Hognestad 1971) Pulmonary embolization is known to induce narrowing of peripheral airways (Thomas *et al*

1964 Nadel *et al* 1964) Furthermore the finding in the third hour post bleeding of an alveolar arterial oxygen tension difference of about 215 mm Hg during oxygen breathing is compatible with a pulmonary vascular shunt fraction in the order of 10 per cent This shunt fraction although small suggests the presence of peripheral airway collapse Both narrowing and collapse of peripheral airways would tend to reduce dynamic lung compliance

The high inverse correlation between changes in compliance and pulmonary arterial pressure most likely reflects a common denominator for the two variables and not any causal relationship

The mean pre bleeding and control value of nonelastic resistance of 22 5 cm H O/l/s is in accordance with previous reports (Nisell and Dubois 1954) Cahill and Byrne (1964) found during shock and concomitant pulmonary hypovolemia a fall in resistance to air flow in the majority of instances Although this was the case also in the present experiments significant changes in this parameter during the 3 h observation period could not be detected We suggest that the moderate changes which did occur mainly reflect variations in the bronchial mucous secretion Such secretion appeared to be somewhat larger in the normotensive control group of animals

This work has been supported through grants from The Norwegian Council on Cardiovascular Diseases This support is gratefully acknowledged The skilled technical assistance of Mrs Kari Tangen is also gratefully appreciated

References

AARSETH P and G Bo Pulmonary blood and extravascular water volumes during prolonged post hemorrhagic systemic hypotension *Acta physiol scand* 1972 *85* 353—361

Bo G A HALGE and J HOGNESTAD Pulmonary hypertension during systemic hypotension Effects of oxygen breathing and of vasodilators *Acta physiol scand* 1972a *86* 460—473

Bo G A HALGE and B A WAALER The relative effects of pulmonary vascular distention and interstitial edema on dynamic lung compliance 1972 b Under preparation

Bo G and J HOGNESTAD Thrombocytes and pulmonary vascular resistance during hemorrhagic hypotension *Acta physiol scand* 1971 *82* 218—228

CAHILL J M and J J BYRNE Ventilatory mechanics in hypovolemic shock *J appl Physiol* 1964 *19* 679—682

GERST P H C RATTENBORG and D A HOLADAY The effects of hemorrhage on pulmonary circulation and respiratory gas exchange *J clin Invest* 1959 *38* 524—538

HENRY J N A H MCARDLE H J SCOTT and F N GURD A study of the acute and chronic respiratory pathophysiology of hemorrhagic shock *J thorac cardiovasc Surg* 1967 *54* 666—6 8

HUGHES R A J MAY and J G WIDDICOMBE The effect of pulmonary congestion and oedema on lung compliance *J Physiol* (Lond) 1958 *142* 306—313

MEAD J and C COLLIER Relation of volume history of lungs to respiratory mechanics in anesthetized dogs *J appl Physiol* 1959 *14* 669—678

MOORE F D et al *Post traumatic pulmonary insufficiency* London W B Saunders Company 1969 1 137 144

NADEL J A H J H COLEBATCH and C R OLSEN Location and mechanism of airway constriction after barium sulfate microembolism *J appl Physiol* 1964 *19* 387—394

NISELL O I and A B DUBOIS Relationship between compliance and FRC of the lungs in cats and measurement of resistance to breathing *Amer J Physiol* 1954 *178* 206—210

PROCTOR H J G S MOSS L D HOMER and B D LITT Changes in lung compliance in experimental hemorrhagic shock and resuscitation *Ann Surg* 1969 *169* 82—92

SALZANO J and F G HALL Effects of a graded hemorrhage on respiratory mechanics *J surg Res* 1961 *1* 48—52

SPELLS K E Comparative studies in lung mechanics based on a survey of literature data *Resp Physiol* 1969/70 *8* 37—57

THOMAS D M STEIN G TANABE V REGE and S WESSLER Mechanism of bronchoconstriction produced by thromboemboli in dogs *Amer J Physiol* 1964 *206* 1207—1212

Acta physiol scand 1973 87 455—464
From the University Institute for Experimental Medicine University of Copenhagen Denmark

The Effects of Amytal and Inactin on Isosmotic Net Fluid Transport in the Rabbit Gall-Bladder In Vitro

By

P CHRISTENSEN L ØSTERGAARD KRISTENSEN and P P LEYSSAC

Received 28 June 1972

Abstract

CHRISTENSEN P L Ø KRISTENSEN and P P LEYSSAC *The effects of amytal and inactin on isosmotic net fluid transport in the rabbit gall bladder in vitro* Acta physiol scand 1973 *87* 455—464

The effects of the oxybarbiturate Amytal and the thiobarbiturate Inactin on isosmotic fluid absorption by the rabbit gall bladder in vitro sac preparation was studied Both barbiturates inhibited net fluid transfer rate The effect was dose-dependent Inactin was a more potent inhibitor than Amytal with effect from the serosal side at concentrations down to 1×10^{-4} M (i e below concentrations obtained in plasma of anesthetized rats) inhibition by Amytal unilaterally was not observed at concentrations below 1×10^{-3} M (a concentration above that of plasma of anesthetized rats) Amytal depressed oxygen consumption in parallel with net fluid transport rate while Inactin was without any significant effect on oxygen consumption in spite of severe inhibition of net fluid transport rate The inhibitory effect of Amytal applied bilaterally was reversible after exposure to the drug for more than 60 min The effect of brief exposure (20 min) to Inactin bilaterally was reversible after exposure for 60 min or more the effect was irreversible in recovery periods of up to 60 min duration Both barbiturates accumulated in the gall bladder tissue with a concentration factor of about 1 5 After washing in barbiturate free Ringer solution (recovery periods) for 45—60 min neither Amytal nor Inactin were detectable in the tissue The sensitivity of the method allowed detection of 0 2 mM Inactin per kg wet weight i e no more than ? of the Inactin accumulated during the experimental period The data suggested that the effects of the two barbiturates were additive

Barbituric acid derivatives oxy as well as *thio* barbiturates are the most common anesthetics used in experimental renal physiology For micropuncture studies in rats the thiobarbiturate Inactin has been the anesthetic preferred by most investigators while only few groups including our own have used the oxy barbiturates sodium amytal or sodium pentobarbital Considerable discrepancies in results on tubular function have been reported from groups using Inactin and oxy barbiturates and it became evident that two distinct functional states were obtained in rats anesthetized with either of these two types of barbiturate A more detailed investigation of the possible influence of the two types of barbiturate on tubular reabsorptive processes was therefore needed Two parallel series of experiments were carried out, an *in vivo* and an *in vitro* study on the relationship between fluid absorption ra the concentration of Inactin or Amytal respectively The results from th

study on rats reported in a preceding paper (Elmer *et al* 1972) showed that proximal as well as distal reabsorptive rates were independent of the level of serum— and tissue barbiturate concentrations in rats anesthetized with Amytal In contrast proximal—but not distal—reabsorptive capacity was depressed and inversely related to the serum Inactin concentration over the range of values obtained in Inactin anesthetized animals

The present paper describes the results from the *in vitro* study The gall bladder *in vitro* sac preparation was chosen as a suitable model for proximal isosmotic fluid transport because of the close similarity in structural as well as functional characteristics between these two epithelia

Marked differences between the effects on the fluid transfer process by Inactin and Amytal were observed Both barbiturates may depress the net absorptive rate of the bladder and both accumulate in the tissue However Inactin is a more potent inhibitor of transepithelial fluid transfer than Amytal causing inhibition at concentration levels equal to those measured in plasma of anesthetized rats in contrast to Amytal Further the inhibitory effect of Inactin was irreversible and without any effect on oxygen consumption (Q_{O_2}) while that of Amytal was reversible and a constant ratio of Q_{O_2}/net fluid transfer rate was maintained independent of the degree of inhibition

Methods

Gall bladders of white female rabbits weighing 2 5—3 0 kg were used The procedure for preparing the gall bladder and for measuring net fluid transport rate gravimetrically has been described in detail previously (Diamond 1964 Frederiksen and Leyssac 1969)

The Ringer solution had the following composition (meq/l) Na 114 7 K 7 0 Ca^{2} 4 0 Mg 2 4 Cl 102 HCO_3 17 5 SO_4 2 4 H_2PO_4 1 2 glutamate 5 0 glucose 1 0 pH was adjusted to 7 35—7 55 by equilibration with 4 % CO_2 and 96 % O_2 at 37 C

Amytal and Inactin Ringers were prepared to give the same Na concentration as that of control Ringers by substituting NaCl for Na barbiturate Otherwise the composition of barbiturate containing Ringer solutions was identical with the control Ringer The barbiturate did not affect the pH significantly Fresh solutions were prepared each day

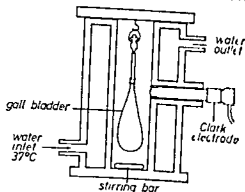
The procedure for measuring the dose response reversibility and oxygen consumption (Q_{O_2}) was as follow The rate of net fluid transfer was measured first in a control period until fluid transfer rate had reached a steady level Q_{O_2} was measured After this control period one of the two barbiturates was applied to the serosal side at the concentration desired when a new steady transport level had been reached and Q_{O_2} measured the barbiturate was added also to the mucosal side at the same concentration When a steady transport level had been reached and Q_{O_2} measured again the gall bladder was returned to the control Ringer solution (both sides) for a recovery period

The reversibility of effect following brief exposure to Inactin was tested in a separate series of experiments After a control period 3 gall bladders were exposed to 3 mM Inactin bilaterally for 20 min and thereafter returned to control Ringer solution For comparison 3 bladders were exposed to 3 mM Inactin bilaterally for 80—100 min before being returned to the control bathing medium

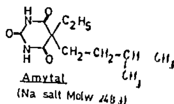
In an additional series of 3 expts 3 mM Amytal or 1 mM Inactin was applied to the serosal side to give about 35 % inhibition When the new steady transport level had been reached the second barbiturate was added to the serosal medium at a concentration which would cause 35 % inhibition when given alone (Amytal 3 mM Inactin 1 mM) Finally a recovery period was obtained

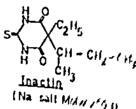
For studies of tissue uptake of the barbiturates from the bathing medium one of the following two experimental procedures was employed

a) After an initial control period one of the two drugs was applied to the medium bilaterally When the new steady transport level had been reached the gall bladder was cut



Fg 1 Schematic presentation of set up for measurement of oxygen consumption





Fg 2 Molecular structure and molecular we ght of Amytal and Inactin

open blotted on filter paper and the muco al layer was separat [illegible] Samples f om the 2 layers were we ghed and analyzed for barbiturate [illegible]

b) After a control per od and an experim ntal per od in which [illegible] bilaterally to the barbiturate the bladder was returned to the [illegible] ments of transport for 45—60 m n dur ng this eco ery peri d [illegible] blotted and the 2 layers separated Samples from the two layers were [illegible] for barbiturate content

Measurements of ox gen onsumpt on we e made with a [illegible] (type E 5046) connected to a Radiometer Acid Ba e analyzer [illegible] simultaneously on a Servo Writ r II (type FS01W5D) [illegible] measurements is shown chemat cally in F g 1 The el [illegible] aerated Ring r solution onta ned n a 28 6 ml clo ed [illegible] thermostatically To obtain in tant m xing of the solution [illegible] the chamber Th oxygen ele trode was al brated with [illegible] 10 S4150) and th a ga m xture containing 4 CO_2 [illegible] equation

$$pO = \frac{(B \; pH \; O) \, x}{100}$$

whe e B is the baromet ic pressure (mm Hg) pH_2O [illegible] (47 1 mm Hg) and x the percentage of O_2 in the gas [illegible]

Amytal and Inactin we mea ured as de cribed previ [illegible] essentially similar to that described by Frey *et al* (1961) [illegible]

The sensiti ity of the method when applied to sm ll sa ples [illegible] g was 0.1 [illegible] of Inactin pe kg of wet weight

TABLE I. Number in parenthesis indicates number of experiments. Data given as mean ± S.D. The p values give the significance level of the difference between control and experimental periods. The effect of amytal was reversible at the significance level of P < 0.01. The effect of inactin was irreversible at the significance level of P < 0.05.

		Amytal					Inactin				
conc. mM	side of application	latency (min.)	time for 1/2 max. inhib.	max. inhib. %	rev.	P	latency (min.)	time for 1/2 max. inhib.	max. inhib.	rev.	P
0.1	serosal								4.5 N.S.		N.S.D.
	bilateral								16.4 N.S.		< 0.05
0.2	serosal						7	20(2)	10.3 ± 3.5		< 0.05
	bilateral							20(3)	25.3 ± 7.5	−	< 0.01
0.5	serosal			0.0 N.S.		N.S.	5	20(4)	21.1 ± 3.5		< 0.02
	bilateral		10	4.1 ± 5.8		N.S.		22(4)	38.9 ± 7.5	−	0.001
1.0	serosal		(3)	1.3 ± 1.1		N.S.	4	26(5)	36.8 ± 1.1		< 0.001
	bilateral		12(5)	23.3 ± 7.9	+	< 0.02		15(4)	54.0 ± 0.7	−	0.001
2.0	serosal	8	22(3)	16.7 ± 6.4		0.05	4	21(3)	38.5 ± 8.5		0.001
	bilateral		13(4)	62.1 ± 6.0	+	0.001		21(2)	62.0 ± 11.0	−	< 0.001
3.0	serosal	9	23(5)	34.4 ± 5.9		< 0.001	5	24(4)	51.5 ± 12.0		< 0.001
	bilateral		14(6)	76.3 ± 4.3	+	0.001		16(7)	63.2 ± 9.8		< 0.001
4.0	serosal	11	30(4)	63.7 ± 10.4		0.001					
	bilateral		14(2)	83.5 ± 4.9	+	< 0.001					
6.0	serosal						5	23(2)	64.6 ± 3.7		< 0.001
	bilateral		13(4)	80.8 ± 3.4		0.001		10(5)	81.2 ± 4.1		< 0.001
8.0	serosal	10	27(3)	80.3 ± 4.0		0.001					
	bilateral		18(2)	86.1 ± 3.8	+	0.001					

Results

Effect on net transcellular fluid transport rate

Amytal as well as Inactin inhibited net fluid transport rate by the gall bladder when applied either unilaterally to the serosal side or bilaterally. The inhibition was dose dependent for unilateral as well as bilateral exposure. The results are summarized in Table I, in which delay time for onset of inhibition, time for half maximum inhibition, maximum inhibition and the degree of reversibility are given.

Fig. 3 and 4 show the dose response curves for Amytal and Inactin respectively, the effects being expressed as a percentage of the control values measured prior to drug application. As seen from Table I and Fig. 3 and 4, the effects of the two barbiturates differed qualitatively as well as quantitatively. There seemed to be no correlation between the latency and the barbiturate concentration, but the delay time for onset of Amytal action was about twice as long as that for Inactin action. Further, the data indicate that Inactin is a more potent inhibitor than Amytal: with unilateral exposure the minimum effective dose of Inactin was 1×10^{-4} M while that of Amytal was 1×10^{-3} M. Since the slopes of the Inactin dose response curves are far less steep than those of the Amytal curves, the differences in concentrations giving 50 % inhibition (I_{50}) were less marked: with unilateral application I_{50} for Inactin was 3×10^{-3} M and that for Amytal 3.5×10^{-3} M; with bilateral exposure I_{50} for Inactin was 9.5×10^{-4} M and that for Amytal 1.7×10^{-3} M.

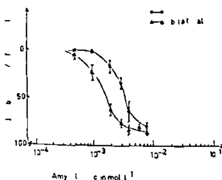


Fig 3

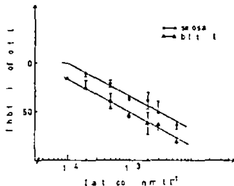


Fig 4

Fig 3 Relationship between medium Amytal concentration and inhibition of net fluid absorption rate after serosal and bilateral application respectively Values are means ± S D

Fig 4 Relationship between medium Inactin concentration and inhibition of net fluid absorption rate after serosal and bilateral application respectively Values are means ± S D

Net transcellular fluid transfer was never completely stopped by the barbiturate concentrations used Neither with Amytal nor with Inactin was the percentage inhibition dependent upon the control transfer rate i e there was no apparent threshold transport level for the inhibition produced by these two barbiturates

Effect on oxygen consumption

Amytal and Inactin had quite different effects on oxygen consumption by the gall bladder A direct linear relationship between net fluid transport rate and Q_{O_2} was apparent at all degrees of inhibition in bladders exposed to Amytal (Fig 5) implying a constant ratio of Q_{O} /transported fluid volume In contrast Inactin had no significant effect on total oxygen consumption (Fig 6) implying an increasing ratio of Q_{O} /transported fluid volume with increasing Inactin concentration

Reversibility

Transport inhibition induced by Amytal bilaterally was reversible at all concentrations studied ($p < 0.01$ 22 expts) independent of the duration of exposure while it was irreversible at all concentrations studied following bilateral exposure to Inactin for more than 60 min ($p < 0.05$ 12 expts) as apparent from Table I However when the bladders were exposed to Inactin for 20 min or less the inhibitory effect of the drug was essentially completely reversible as apparent from Fig 7

Drug interaction

In 3 expts the possible interaction between the 2 barbiturates was tested The results suggest that the inhibitory effects of unilateral application of 3 mM Amytal and 1 mM Inactin were additive and partly reversible (Fig 8)

It was observed that Amytal nearly always produced a relaxation of the bladder wall while the effect of Inactin on smooth muscular tone was more inconsistent



Fig 5

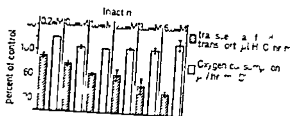


Fig 6

Fig 5 The effects of increasing serosal Amytal concentrations on net fluid tran port rate and on oxygen consumption Values are means ± S.D

Fig 6 The effects of increasing serosal Inactin concentrations on net fluid tran port rate and on oxygen consumption Values are means ± S.D

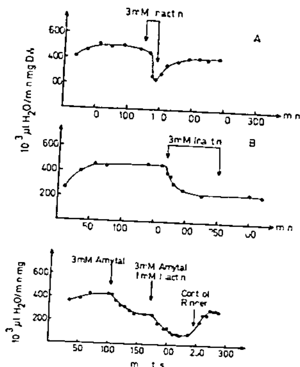


Fig 7 Records of 2 representative expts showing the effect on net fluid absorption rate of 3 mM Inactin applied bilaterally in A for 20 min in B for 80 min The time interval between the arrows indicates the duration of Inactin exposure

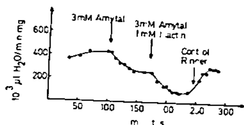


Fig 8 Record of an experiment demonstrating additive effects of 3 mM Amytal and 1 mM Inactin on net fluid absorption rate

some bladders contracted others relaxed while some were entirely unaffected When in the first period Inactin was applied and a contraction appeared the bladder wall then relaxed in the following period both barbiturates being present in the bathing medium

Tissue binding of the barbiturates

Tissue uptake was studied in a series of experiments in order to test whether or not there was any difference in the binding to the serosal or mucosal layer of the gall bladder of the two barbiturates investigated

As seen from Fig 9 and Fig 10 both barbiturates accumulated to about the same degree in the mucosal and the serosal layer of the bladder wall The concentrating

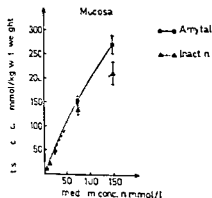


Fig 9

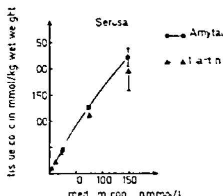


Fig 10

Fig 9 *Mucosal* layer barbiturate concentration plotted against medium barbiturate concentration.

Fig 10 *Serosal* layer barbiturate concentration plotted against medium barbiturate concentration

factor was about 1 5 for both drugs in the serosal wall at all concentrations studied and about 1 5 for Inactin and 2 0 for Amytal in the mucosal layer Neither of the 2 barbiturates were detectable after washing for 45—60 min during the recovery period in barbiturate free control medium In Amytal treated gall bladders transport rate always returned towards control levels at the end of the recovery period while transport rate of bladders exposed to Inactin remained irreversible at the inhibited level during the entire recovery period in all experiments after prolonged exposure to the drug

Discussion

The present investigation shows that both barbiturate studied may depress trans epithelial transport for solutes and water by the gall bladder *in vitro* preparation The effect was dose dependent Further Inactin appears to be a more potent inhibitor of the fluid transport rate than Amytal as evidenced by the observations firstly that the minimal effective Inactin concentration was 10 times lower than that of Amytal and secondly that the latency for onset of inhibition was only half that of Amytal

The present results are in close accordance with and substantiate those obtained in the parallel *in vivo* study on tubular reabsorption rate in Amytal and Inactin anesthetized rats respectively (Elmer *et al* 1972) Medium Amytal concentrations equal to those in serum of anesthetized rats (about 5×10^{-4} M *i e* 12 5 mg %) were without any inhibitory effect on the present *in vitro* transport rate by the gall bladder when applied either unilaterally or bilaterally In contrast medium Inactin concentrations equal to those in serum of anesthetized animals (about 5×10^{-4} M) inhibited net fluid transport rate by 21 ± 3 5 % when applied unilaterally to the

serosal bathing medium and by 39±7 5 % when applied bilaterally Comparing the mean proximal reabsorption rate of 0 72 ml min⁻¹ g KW⁻¹ in Amytal anesthetized rats with the mean value of 0 46 ml min⁻¹ g KW⁻¹ obtained in rats anesthetized with Inactin (Elmer *et al* 1972) indicates that Inactin (5×10⁻⁴) had depressed proximal reabsorption rate by 36 % *in vivo*

The irreversibility of the Inactin effect as opposed to the reversible effect of Amytal observed in the present *in vitro* investigation may have a bearing on some *in vivo* observations in rats The anesthetic effect of a single Amytal dose is relatively brief and repeated supplementary doses are required to maintain adequate anesthesia during an experiment With Inactin only a single dose is required for maintenance of anesthesia throughout an experiment actually a rat anesthetized with a single dose of Inactin will remain asleep for several days until it dies (unpublished observation) Further the results obtained *in vivo* by Elmer *et al* (1972) indicated that the rate limiting factor for proximal reabsorption rate under Inactin anesthesia could not be a direct function of the plasma concentration at the moment of measurement of the tubular transport rate even though a correlation was apparent In view of the irreversibility of the Inactin effect and its time dependency observed in the present *in vitro* experiments it is quite possible that a high plasma concentration obtained initially—or rather a function of a time integral of the initial plasma concentration—may be rate limiting throughout an *in vivo* experiment even though a fairly low plasma concentration might exist at the time of the actual measurements

Both barbiturates accumulated in the gall bladder tissue—in the serosal as well as in the mucosal layer—to about the same degree as in the renal cortex *in vivo* (*cf* Elmer *et al* 1972) The data from the *in vivo* investigation indicated that the proximal reabsorption rate was not determined by the over all cortical tissue barbiturate concentration in rats anesthetized either with Amytal or Inactin The present study is not at variance with this conclusion Regarding the Inactin effect on transport rate it remained unaffected in the recovery period in which accumulated tissue Inactin was washed out The sensitivity of the analytical method for such small tissue samples (10 mg) as obtained in the present study allows the conclusion that the bladder tissue did not contain more than 0 12 mM Inactin per kg wet weight of tissue at the end of the recovery period *i e* whilst the inhibition remained unchanged the bladder tissue contained at most 2 % of the Inactin present at the end of a period of incubation with 1 mM Inactin in the medium

Besides the difference in potency and in reversibility of the inhibitory effect the two barbiturates also differed in their effect on oxygen consumption At low concentrations (⩽ 1 mM) Amytal was without any effect either on net fluid transport or oxygen consumption With increasing concentrations of Amytal net transport and Q_{O_2} decreased in parallel In contrast Inactin did not depress oxygen consumption in spite of marked inhibition of fluid transport rate Amytal inhibits the respiratory chain in isolated mitochondria (Ernster *et al* 1955) by inhibiting electron transfer between NADH and flavoproteins (Chance 1956) In the toad bladder Amytal

also depressed oxygen consumption as well as sodium transport as measured by the short circuit current (SCC) (Hidalgo *et al* 1966) However bilateral exposure of the toad bladder to 4×10^{-3} M of Amytal completely abolished oxygen consumption while SCC was only inhibited by 80 % In the isolated gall bladder a much higher concentration of Amytal was required to reach comparable degree of inhibition of oxygen consumption 8×10^{-3} M of Amytal applied to the serosal bath depressed fluid transfer rate and oxygen consumption by 80 % and 70 % respectively Bilateral application of Amytal at this concentration inhibited net transport by 86 % and Q_{O_2} by 91 % (not tabulated) Thus even though a quantitative difference apparently exists between the toad bladder and the gall bladder the Amytal effects may be explained by its action on the respiratory chain

The lack of inhibition of oxygen consumption by Inactin at concentrations effective in depressing net transcellular fluid transport is more difficult to interpret Several alternatives appear open The effect suggests some kind of uncoupling In intact cells in which oxygen consumption is ADP controlled uncoupling of oxydative phosphorylation by 2 4 dinitrophenol (DNP) results in augmented oxygen consumption Q_{O_2} remained unaffected by Inactin in the present experiments indicating that the effect is not a simple DNP like uncoupling of mitochondrial oxydative phosphorylation However it has been shown that the thiobarbiturates thiopentone thiobarbitone and butalitone uncouple oxydative phosphorylation like DNP in ADP limited isolated miochondria (Aldridge 1960 1962) but inhibit respiration in ADP stimulated miochondria (Aldridge 1960 Chance *et al* 1963) Thus it remains possible that Inactin may have dual effects uncoupling of oxydative phosphorylation while at the same time inhibiting the respiratory chain Alternatively the effect might be at the level of an ATP ase by uncoupling transport from energy expenditure of the pump

A different line of interpretation would be by postulating a change in membrane permeability by Inactin In this case one would have to postulate that Inactin is without any direct effect either on ultilization of mitochondrial energy production or he pump while Amytal at similar concentrations inhibits the respiratory chain First a decreased net tran port rate in the presence of unchanged oxygen consumption would result if the passive backflux of solute and water is increased by an increase in the permeability of a shunt path between the epithelial cells A second alternative would be that Inactin might increase the ion permeability of the epithelial cell membrane resulting in an increased Na/K concentration ratio intracellularly Several lines of evidence obtained from isosmotically transporting epithelia (*e g* proximal tubule and gall bladder) had indicated two distinct pump mechanisms a homocellular Na K pump responsible for the maintenance of normal Na K concentrations intracellularly and a NaCl or volume pump concerned with regulation of cell volume and/or transcellular fluid transport (Bojesen and Leyssac 1965 Whittembury 1968 Whittembury and Fishman 1969 MacKnight 1968 Maude 1969 1970 Frederiksen and Leyssac 1969) Further Frederiksen and Leyssac (1969) presented evidence suggesting that a high intracellular Na/K concentration ratio

would inhibit the transcellular pump mechanism (net fluid transfer rate) while at the same time stimulating the Na K pump and thereby the basal oxygen consumption Thus an unchanged total oxygen consumption in the presence of Inactin might be the result of simultaneous stimulation of the basal Q_{O_2} and depression of the suprabasal Q_{O_2} linked to transcellular fluid transfer It has been shown that creases Na permeability in the toad bladder Small concentrations (0 2 mM) in thiobarbiturates thiopentone and butyl crotyl thiobarbiturate applied bilaterally increased while higher concentrations (1 0 and 2 0 mM) decreased Na transport as measured by the SCC (Andersen 1970) In view of these observations the last mentioned interpretation of the Inactin effect might seem the more attractive

The present study was supported by grants from the Danish State Medical Research Council and Ebba Celinders Legat

References

ALDRIDGE W N Barbiturates and the respiratory chain In *Enzymes and drug action* J L Mongar and A V S de Reuck Editors Ciba foundation symposium J and A Churchill Ltd London 1962 162—169

ALDRIDGE W N and V H PARKER Barbiturates and Oxidative Phosphorylation *Biochem J* 1960 *76* 47—56

ANDERSEN N B The effect of a convulsant (butyl crotyl thiobarbituric acid) and an anaesthetic (thiopental) on the sodium fluxes in toad bladder *J Pharmacol exp Ther* 1970 *173* 317—322

BOJESEN E and P P LEYSSAC The kidney cortex slice technique as a model for sodium transport in vivo *Acta physiol scand* 1965 *65* 20—32

CHANCE B Interaction of Adenosinediphosphate with the Respiratory Chain In *Enzymes Units of Biological Structure and function* O H Gaebler Editor Henri Ford Hospital International Symposium Acad Press Inc New York 1956 447—463

CHANCE B and K G HOLLUNGER Inhibition of electron and energy transfer in mitochondria *J biol Chem* 1963 *278* 418—431

DIAMOND J M The mechanism of isotonic water transport *J gen Physiol* 1964 *48* 15—42

ELMER M P C ESKILDSEN L Ø KRISTENSEN and P P LEYSSAC A comparison of renal function in rats anesthetized with Inactin and Sodium Amytal *Acta physiol scand* 1972 *86* 41—58

ERNSTER L O JALLING H LOW and O LINDBERG Alternative pathway of mitochondrial DPNH oxidation studied with Amytal *Exp Cell Res* 1955 *Suppl 3* 124—132

FREDERIKSEN O and P P LEYSSAC Transcellular transport of isotonic volumes by the rabbit gall bladder in vitro *J Physiol* (Lond) 1969 *201* 201—224

FREY H H A DOENICKE and G JAGER Quantitative Bedeutung der Desulfurierung im Stoffwechsel von Thiobarbituraten *Med Exp* 1962 *4* 243—250

HIDALGO C and M CANESSA FISCHER Sodium transport inhibition by selective Mitochondrial inhibitors in the urinary bladder of the toad *J Cell comp Physiol* 1966 *68* 185—196

MACKNIGHT A D C Water and electrolyte contents of rat renal cortical slices incubated in potassium free media and media containing ouabain *Biochim biophys Acta* (Amst.) 1968 *150* 263—270

MAUDE D L Effects of K and ouabain on fluid transport and cell Na in proximal tubule in vitro *Amer J Physiol* 1969 *216* 1199—1206

MAUDE D L Mechanism of salt transport and some permeability properties of rat proximal tubule *Amer J Physiol* 1970 *218* 1590—1595

WHITTEMBURY G Na and water transport in kidney proximal tubular cells *J gen Physiol* 1968 *51* 303—314

WHITTEMBURY G and J FISHMAN Relation between cell Na extrusion and transtubular absorption in the perfused toad kidney The effect of K Ouabain and Ethacrynic acid *Pflugers Arch ges Physiol* 1969 *307* 138—153

Acta physiol scand 1973 87 465—473
From the Department of Physiology University of Goteborg Sweden

Glycogen Synthesis in Rat Diaphragm in Vivo a Biphasic Effect of Insulin on Glycogen Synthetase Enzyme

By

STEN ADOLFSSON

Received 11 July 1972

Abstract

ADOLFSSON S *Glycogen synthesis in rat diaphragm in vivo a biphasic effect of insulin on glycogen synthetase enzyme* Acta physiol scand 1973 *87* 465—473

During enhanced glycogen synthesis an increase in the activity of the enzyme rate limiting for glycogen synthesis is expected This enzyme glycogen synthetase (UDP glucose α 1 4 glucan α 4 glucosyltransferase EC 2 4 1 11) can be activated by transformation of its D form to I form Insulin is known to stimulate glycogen synthesis in skeletal muscle However Sovik did not find any change in I form in the rat diaphragm 30—240 min after an intraperitoneal (i p) injection of insulin *although glycogen synthesis was highly stimulated (O Sovik Acta physiol* scand 1966 *68* 246—254) In the present study glycogen content and synthetase enzyme activity were measured in rat diaphragms 10—120 min after i p injection of insulin Phosphorylase enzyme activity was also measured Insulin administration produced a biphasic change in the synthetase enzyme compared to controls there was an increase of the % I form after 10 and 20 min and a decrease after 50 80 and 120 min Phosphorylase enzyme activity was unaffected Glycogen increased continuously up to 120 min Thus the increase in I form was shown to be a transient effect of insulin In Sovik s experiment this increase had probably subsided at the time of the first measurement To account for the increase in glycogen when the % I form was decreased an activation of the synthetase enzyme through an increase of its activator glucose 6 phosphate may be considered

The glycogen synthetase enzyme (UDP glucose α 1 4 glucan α 4 glucosyltransferase EC 2 4 1 11) is considered rate limiting for glycogen synthesis in skeletal muscle and other tissues (Villar Palasi and Larner 1961) This enzyme consists of two interconvertible forms (Friedman and Larner 1963) the I form [independent of glucose 6 phosphate (glucose 6 P)] and the D form (dependent on glucose 6 P) A transformation of D form to I form will increase the activity of the enzyme in the tissue Changes in the intracellular concentration of certain metabolites will also affect the activity of the synthetase enzyme e g glucose 6 P activates the enzyme (Piras *et al* 1968)

Insulin increases the penetration of glucose into skeletal muscle and also directs glucose towards glycogen formation by increasing the activity of the synthetase enzyme The mechanisms by which insulin activates this enzyme are not yet

clarified It has been shown (Villar Palasi and Larner 1961) that insulin activates the synthetase enzyme by causing a transformation of D form to I form in rat diaphragm muscle incubated *in vitro* for 30 min with the hormone Similar activation of the enzyme has been demonstrated *in vivo* in hind leg muscles of rats 5—30 min following an intraperitoneal (i p) injection of insulin (Goldberg *et al* 1967) Søvik (1966) however, failed to find such a D to I form transformation of the enzyme *in vivo* in the diaphragm muscle of rats 30—240 min following an i p injection of insulin In his experiments glycogen synthesis in the diaphragm was highly stimulated for the first 120 min following injection of the hormone indicating that the synthetase enzyme must have been activated during that period Since he found an increase in the glucose 6 P level in the diaphragm Søvik concluded that insulin activates the enzyme *in vivo* by increasing the intracellular level of glucose 6 P

Probably both the increase in I form and the activation through increased level of glucose 6 P constitute physiological mechanisms by which insulin increases synthetase enzyme activity The increase in I form may only be an initial and transient effect of insulin which would not have been detected in Søvik s experiments because the first measurements of enzyme activity were not made until 30 min after the animals were treated with insulin This hypothesis was tested in the present study Glycogen content and synthetase enzyme activity were measured in rat diaphragm muscle 10—120 min following an i p injection of insulin Phosphorylase enzyme activity was also measured

Methods

Experimental procedure

Male rats of the Sprague Dawley strain weighing 50—65 g were used They were maintained on a semi synthetic diet (Gustafsson 1959) and tap water and had no access to food 18—24 h before the experiment Porcine insulin in 0 5—0 6 ml 0 9 % NaCl was injected i p to the right of the umbilicus in a dose of 10 mU/100 g b w Control animals were injected with a similar volume of 0 9 % NaCl

The animals were killed by cervical fracture 10—120 min after the injection The abdominal aspect of the diaphragm was gently exposed A piece of the right half of the diaphragm was then cut out with the use of a fine pair of scissors (originally designed for iridectomy) This piece of muscle (50—60 mg) was gently blotted on filter paper and divided into two parts which were rapidly weighed on a torsion balance One piece was placed into liquid nitrogen (for subsequent determination of enzyme activity) and the other into 5 M KOH (for glycogen determination) The first muscle was always frozen within 1 min after sacrifice of the animal When the tissue was handled in this way reproducible results were obtained for glycogen and synthetase enzyme activity but not for phosphorylase enzyme activity The percentage of phosphorylase enzyme present as a form was high and variable (40—70 %) This is probably due to activation of the a form during excision of the muscle from the animal Similar variable results were obtained when the muscles were excised from animals anesthetized with Nembutal (60 mg/kg i p) However if a pre weighed muscle was incubated for 30 min as a cut diaphragm preparation in Krebs Ringer bicarbonate buffer pH 7 4 (gassed with a 95 5 O_2 CO_2 mixture and containing glucose 2 5 mg/ml) and then immediately homogenized the per cent of a form was lower and the values more reproducible This procedure was therefore used in the experiments dealing with the phosphorylase enzyme

Determination of glycogen content synthetase enzyme activity and phosphorylase enzyme activity

Glycogen was isolated from 20—30 mg muscle by boiling the tissue for 30 min in 0 4 ml 5 M KOH and by precipitating the glycogen overnight at 4 C in 66 % ethanol 60 mM

Na_2SO_4. The precipitate was washed twice in 1 ml 66 % ethanol, dried and dissolved in 0.4—0.8 ml water. Aliquots of 0.1 ml were added to 1.5 ml reaction mixture containing a commercial α-1,4-α-1,6-amylase to hydrolyse the glycogen into glucose and the usual reagents of the glucose oxidase method (Bergmeyer and Bernt 1963) for spectrophotometric determination of glucose. For each muscle two or three such aliquots were analyzed. Rabbit liver glycogen was used as standard. Glycogen values are expressed as mg glycogen/g wet weight.

Glycogen synthetase activity was measured as radioactivity incorporated into glycogen from UDP-glucose-^{14}C (U) (Villar-Palasi et al. 1966). The piece of diaphragm frozen in liquid nitrogen was homogenized (25 mg/ml) in Tris buffer pH 7.8 (Tris 50 mM, EDTA 5 mM, NaF 10 mM) using an all glass homogenizer which was kept in ice-water. After centrifugation at ° C for 30 min at 5000 × g aliquots of 50 μl of the supernatant were incubated with 50 μl reaction mixture for 10 min at 30 °C. The reaction was linear during 0—15 min of incubation. Incubations with and without glucose-6-P were made in duplicate. The Tris buffer described above was used; reactants were present at the following final concentrations: UDP-glucose 5 mM, UDP-glucose-^{14}C 0.5 μCi/ml, glycogen 1 % and glucose-6-P 10 mM. After incubation the glycogen was isolated and assayed for radioactivity as described by Thomas et al. (1968). Enzyme activity is expressed as U/g wet weight (1 U = 1 μmol glucose incorporated into glycogen/min). The activity in the presence of 10 mM glucose-6-P is referred to as total enzyme activity (I+D) form and the activity in the absence of glucose-6-P as I form. I form activity is also expressed as a percentage of total enzyme activity, % I form.

Glycogen phosphorylase activity was measured in the direction of glycogen degradation essentially as described by Bueding et al. (1962). A non-centrifugated muscle homogenate of 10 mg muscle in 1 ml citrate buffer pH 6.7 was used. The tissue was homogenized in an all glass homogenizer kept in ice-water. The amount of glucose-1-phosphate released from glycogen in the assay mixture during 10 min of incubation at 37 °C was estimated spectrophotometrically at 340 nm using NADP and the appropiate enzymes.

Enzyme activity is expressed in U/g wet weight (1 U = 1 μmol glucose-1-phosphate released from glycogen/min). The activity in the absence of added 5'-AMP (a form) is expressed as a percentage of the activity in the presence of 1 mM 5'-AMP (a+b forms) and referred to as % a form.

Chemicals

All chemicals were of analytical grade and were used without further purification. Glycogen (rabbit liver, type III) and α-1,4-α-1,6-amylase (amyloglucosidase grade 2) were obtained from Sigma (St. Louis, USA). The reagents for the glucose oxidase method and the hexokinase-glucose-6-phosphate dehydrogenase method for glycogen and blood glucose determination respectively were purchased as test kits from Boehringer GmbH (Mannheim, Germany). The phosphoglucomutase and the NADP were also from this company. UDP-D-glucose-^{14}C (U) was obtained from NEN Chemicals (Boston, USA). Porcine insulin No. o lot no. S 23267 (10-times recrystallized) was kindly provided by Novo Research Laboratories (Copenhagen, Denmark).

Statistical analyses

Mean values are given ± standard error of the means (S.E.). The one-way analysis of variance was used to reveal significant differences (Woolf 1968). Analyses comparing mean values of treatment groups with that of the control group were performed according to Dunnett's procedure (Dunnett 1955). In experiments with equal group sizes means were compared according to the Student-Newman-Keuls test (Woolf 1968). A p-value less than 0.05 is considered significant in this study.

Results

Influence of insulin on glycogen content

Intraperitoneal injection of insulin (10 mU/100 g b.w.) very markedly enhanced the accumulation of glycogen in the diaphragm muscle (Fig. 1). This effect was significant after 10 min. There was a continuous accumulation of glycogen up to 120 min, which was the longest period investigated. The rate of accumulation was higher during the first 50 min after insulin injection than during the subsequent 50—120 min, 94 and 52 μg glycogen/min × g wet weight, respectively. Five min after insulin

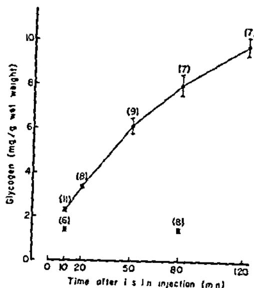


Fig 1

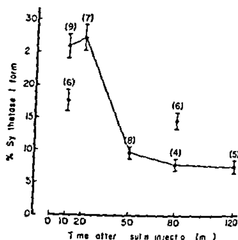


Fig 2

Fig 1 Glycogen content in rat diaphragm muscle after intrapentoneal injection of insulin. Insulin was injected intrapentoneally (10 mU/100 g body weight) into 60 g male rats fasted for 18 h At the periods indicated the right hemidiaphragm was removed and assayed for glycogen as described in *Methods* Compiled data from two experiments are shown Figures within brackets indicate the number of rats in the groups Two standard errors are indicated by the length of the vertical bars The two solitary points show values from control rats injected with saline only

Fig 2 Per cent I form in the synthetase enzyme of rat diaphragm muscle after intrapentoneal injection of insulin Synthetase enzyme activity was determined as described in *Methods* The two solitary points show values of control rats injected with saline There was no significant difference between these two values For further information see legend to Fig 1

njection the glycogen content was not significantly increased (control muscles 1 72 ± 0 08 mg glycogen/g wet weight insulin stimulated muscles 1 97 ± 0 09 5 rats in each group)

Influence of insulin on glycogen synthetase enzyme activity

The activity of the synthetase enzyme after an i p injection of insulin to the rat is illustrated in Fig 2 and Table I Compared to controls there was an increase in % I form at 10 and 20 min This initial increase was followed by a decrease in % I form Lower values of % I form compared to controls were found 50 80 and 120 min after insulin injection Thus a biphasic pattern in the % I form of the enzyme was found with insulin

Total enzyme activity (I + D) form was significantly affected by insulin 120 min after its injection at which time there was a decrease This decrease is not due to an increase in the amount of tissue water since no change was observed in tissue water content 10, 50 and 120 min after insulin injection compared to controls injected with saline 10 min prior to sacrifice After drying pre weighed muscle pieces to constant

TABLE I Glycogen synthetase enzyme activity in rat diaphragm muscle after intraperitoneal injection of insulin

Synthetase activity (U/g wet weight)	Control	Minutes after insulin injection 10	20	50	80	120
I form	0.23±0.02	0.37±0.06*	0.36±0.03*	0.12±0.01	0.09±0.01	0.08±0.01*
(I+D) form	1.42±0.02	1.30±0.10	1.31±0.04	1.23±0.05	1.14±0.05	1.05±0.06*
I form	15.8 ±1.1	25.9 ±1.7**	27.2 ±1.9**	9.9 ±0.8*	8.0 ±0.8	7.6 ±1.0**
n	12	9	7	8	4	5

Synthetase enzyme activity was determined as described in *Methods* Control rats were injected with saline only 10 or 80 min prior to sacrifice Mean values±S E are given n indicates number of rats in each group In all 3 sets of data analysis of variance revealed significant differences ($p <$ 0.01) Dunnett's procedure was used to compare means with control means a significant difference of $p < 0.05$ is marked as * and of $p < 0.01$ as **

TABLE II Glycogen phosphorylase enzyme activity in rat diaphragm muscle after intraperitoneal injection of insulin

Phosphorylase activity (U/g wet weight)	Control	Minutes after insulin injection 10	50
a form	2.01±0.23	2.15±0.03	2.07±0.13
(a+b) form	21.3±1.6	23.0±0.8	21.0±0.4
a form	9.6±0.9	9.7±0.2	10.3±0.6
n	5	4	4

Ten and 50 min after insulin injection cut diaphragms were removed and incubated *in vitro* for 30 min Control muscles were taken from rats injected with saline 50 min before sacrifice After incubation phosphorylase enzyme activities were measured For further information see *Methods* Mean values±S E are given n indicates number of rats in each group Analysis of variance revealed no difference in the 3 sets of data

weight in a vacuum oven at 100° C the following values for total tissue water (ml/100 g wet weight) were calculated controls 77.4±0.4 insulin 10 min 78.0±0.6 insulin 50 min 76.2±1.0 and insulin 120 min 77.4±0.4 5 muscles in each group

Influence of insulin on phosphorylase enzyme activity

As described in *Methods* the phosphorylase enzyme is markedly influenced by the dissection of the tissue Due to lack of a good technique to freeze the diaphragm *in situ* the tissue was incubated as a cut diaphragm preparation to stabilize the enzyme after the dissection With this technique values for % a form were obtained that are comparable to those found in resting rat leg muscle which can be frozen *in situ* (Posner *et al* 1965) No effect of insulin was found on the two forms of the enzyme (Table II) During the incubation period glycogen content continued to inc in muscles from insulin injected rats indicating a prevailing effect of insulin

Influence of insulin on blood glucose concentration

In a separate series of experiments, rats were decapitated 10 50 and 120 min after the injection of insulin (10 mU/100 g b w) Control rats were injected with saline 10 min prior to death There were 5 rats in each group Duplicate samples of 5 μl of blood were taken immediately following decapitation, and glucose was then determined with the coupled hexokinase glucose 6 phosphate dehydrogenase reaction (Stork and Schmidt 1968) The following levels of blood glucose (mg glucose/100 ml blood) were found control 78 ± 3 insulin 10 min 69 ± 3 insulin 50 min 64 ± 5 and insulin 120 min 53 ± 2 The levels 50 and 120 min after insulin are significantly lower than control but the level after 10 min is not In previous studies there was no significant decrease in blood glucose level 60 and 120 min after i p injection of a similar dose of insulin (10 mU/100 g b w) (Sovik 1966 Rafaelsen 1964) This discrepancy may be due to differences in age of the animals used (60 g rats in the present study compared to 80—160 g rats in the studies cited)

Discussion

In the present study insulin was found to increase the I form of the glycogen synthetase enzyme in rat diaphragm *in vivo* This was observed 10 and 20 min after injection of the hormone but not 50 80 and 120 min after injection In the experiments by Sovik (1966) previously cited no change in I form was found after injection of insulin This discrepancy can be explained by the fact that in his experiments enzyme activity was first measured 30 min after injection of insulin by what time the transitory increase in I form had probably subsided

The increase in the I form of the synthetase enzyme during insulin stimulation *in vivo* has been demonstrated previously in rat hind leg muscle 5—30 min after i p injection of the hormone (Goldberg *et al* 1967) The increase was of approximately the same magnitude (50 %) as in the present study but despite the increase in enzyme activity there was no increase in glycogen in the tissue Furthermore the dose of insulin injected (2—4 IU/kg b w) produced hypoglycemia In the rat diaphragm muscle glycogen synthesis is very markedly enhanced after i p injection of insulin at a dose (0 1 IU/kg b w) that either does not induce hypoglycemia (Sovik 1966 Rafaelsen 1964) or produces only a very slight lowering of blood glucose concentration as seen in the present study Thus this preparation is well suited for *in vivo* studies of the influence of insulin on glycogen synthesis

It was very interesting to note that the rapid and transitory increase in the % I form of the synthetase enzyme due to insulin stimulation was followed by a second phase (50—120 min after insulin) during which glycogen continued to increase although the I form was decreased compared to controls This decrease in I form during the second phase is probably due to the high glycogen levels in the tissue at that time It has been shown with the rat diaphragm *in vitro* that there is an inverse relationship between the glycogen level and the I form of the enzyme (Danforth 1965) This relationship is shifted by insulin to give a higher level of

I form for a given level of glycogen Similarly, in the present study it was found that the % I form decreased as the glycogen level increased during insulin stimulation

At the longest periods of insulin stimulation the decrease in synthetase I form was associated with a minor decrease in total enzyme activity This minor decrease does not necessarily implicate a simultaneous lowering of the D form As pointed out (Mersmann and Segal 1967) the I form is measured more effectively than the D form also when saturating concentrations of UDP glucose glycogen and glucose 6-P are used in the synthetase assay system In rat skeletal muscle the V_{max} value of the I form is slightly higher than that of the D form (Villar Palasi *et al* 1966) Therefore a small decrease in total enzyme activity is expected when there is a pronounced conversion of I form to D form This has also been reported for rat diaphragm muscle during stimulation by epinephrine *in vitro* (Craig and Larner 1964)

In order to explain the increase in glycogen during the second phase of insulin timula ion when the active I form of the rate limiting synthetase enzyme is de pressed more recent studies on the influence of different metabolites on the activity of the synthetase enzyme must be considered It has been shown that both forms of the enzyme are inhibited by physiological concentrations of ATP ADP and inorganic phosphate (Piras *et al* 1968) Inhibition of the I form is reversed by increasing glucose 6 P within the physiological range The inhibition of the D form will only be reversed to a minor extent however unless very high levels of glucose 6 P are reached The levels of ATP ADP and inorganic phosphate are stable under aerobic conditions and not influenced by insulin (Piras and Staneloni 1969 Larner *et al* 1960) Sovik (1966) has reported an increase of glucose 6 P in rat diaphragm 150 min after insulin injection It is therefore reasonable to assume that during the econd phase of insulin stimulation the synthetase enzyme is activated metabolically by an increased level of glucose 6 P Probably this kind of activation is present also initially during insulin stimulation In the literature there are other studies indicating that glycogen synthesis can be accelerated at unchanged levels of I form (Adolfsson and Ahren 1968 1970 Hultman 1967 Piras and Staneloni 1969)

The possibility that the increase in glycogen during insulin stimulation is partly due to an inhibition of phosphorylase enzyme activity was also considered For technical reasons phosphorylase enzyme activity could not be measured directly on the excised diaphragm muscle but was measured after the tissue was incubated *in vitro* for 30 min During this incubation glycogen was further increased in muscles from rats injected with insulin There were no differences in the level of phosphory lase a form between muscles from control and insulin injected rats This is in agree ment with a previous report on the effect of insulin *in vitro* on rat diaphragm (Torres *et al* 1968) In the same report insulin was shown to decrease the elevation of a form induced by epinephrine It can be argued that insulin may depress the a form in the rhythmically contracting diaphragm muscle *in vivo* and that this effe will not be seen when the muscle is incubated *in vitro* since it does not un rhythmic contraction during incubation However insulin does not de

a form in the rhythmically contracting heart muscle (Williams and Mayer 1966 Adolfsson et al 1972) The phosphorylase enzyme is also subjected to regulation by certain metabolites although this metabolic influence is of little importance under aerobic conditions (Morgan and Parmeggiani 1964) Thus there is no obvious indication of an inhibition by insulin of phosphorylase enzyme activity

To conclude an *in vitro* effect of insulin on the synthetase enzyme of the rat diaphragm was observed There was a biphasic response of the I form of the enzyme with a transient initial increase followed by a decrease Glycogen content increased continuously during both phases

The author wishes to thank Professor K. Ahren and Professor J Kostyo for valuable suggestions concerning the manuscript Thanks are also due to Dr J Schlichtkrull at Novo Research Laboratories for the kind gift of porcine insulin and to Dr G Selstam for information of the details of the phosphorylase enzyme analysis

Skilled technical assistance was given by Miss Marianne Dahlberg Miss Eva Weiler and Miss Britt Marie Johansson Economical support for this study was provided by M Bergvalls foundation R. and T Soderbergs foundations Svenska Sallskapet for Medicinsk Forskning and the Faculty of Medicine University of Goteborg

References

ADOLFSSON S and K AHREN Biphasic action of testosterone on muscle glycogen synthetase *Acta physiol scand* 1968 74 30A—31A

ADOLFSSON S and K AHREN Early and late effects of insulin on muscle glycogen synthesis In *International Congress Series No* 209 Excerpta Medica Foundation Amsterdam 1970 p 51

ADOLFSSON S O ISAKSSON and A HJALMARSON Effect of insulin on glycogen synthesis and synthetase enzyme activity in the perfused rat heart *Biochim biophys Acta* (Amst) 1972 279 146—156

BERGMEYER H U and E BERNT D glucose Determination with glucose oxidase and peroxidase In *Methods of Enzymatic Analysis* Ed Bergmeyer H U Verlag Chemie Weinheim 1963 pp 123—130

BUEDING E E BULBRING H KURIYAMA and G GERCKEN Lack of activation of phosphorylase by adrenaline during its physiological effect on intestinal smooth muscle *Nature* (Lond) 1962 *196* 944—946

CRAIG J W and J LARNER Influence of epinephrine and insulin on uridine diphosphate glucose-α glucan transferase and phosphorylase in muscle *Nature* (Lond) 1964 *202* 971—973

DANFORTH W H Glycogen synthetase activity in skeletal muscle Interconversion of two forms and control of glycogen synthesis *J biol Chem* 1965 *240* 588—593

DUNNETT CH Comparing several treatments with a control *J amer statist Ass* 1955 *50* 1096—1121

FRIEDMAN D L and J LARNER Studies on UDPG α glucan transglucosylase III Interconversion of two forms of muscle UDPG-α glucan transglucosylase by a phosphorylation-dephosphorylation reaction sequence *Biochemistry* 1963 2 669—675

GOLDBERG N D C VILLAR PALASI H SASKO and J LARNER Effects of insulin treatment on muscle 3 5-cyclic adenylate levels *in vivo* and *in vitro* *Biochim biophys Acta* (Amst.) 1967 *148* 665—672

GUSTAFSSON B E Light weight stainless steel systems for rearing germfree rats *Ann N Y Acad Sci* 1959 *78* 17—28

HULTMAN E Studies on muscle metabolism of glycogen and active phosphate in man with special reference to exercise and diet *Scand J clin Lab Invest* 1967 *19* Suppl 94 p 78

LARNER J C VILLAR PALASI and D J RICHMAN Insulin stimulated glycogen formation in rat diaphragm. Levels of tissue intermediates in short time experiments *Arch Biochem* 1960 *86* 56—60

MERSMANN H and H SEGAL An on-off mechanism for liver glycogen synthetase activity *Proc nat Acad Sci* (Wash.) 1967 *58* 1688—1695

MORGAN H. E and A PARMEGGIANI Regulation of glycogenolysis in muscle III Control of muscle glycogen phosphorylase activity *J biol Chem* 1964 *239* 2440—2445

PIRAS R L B ROTHMAN and E CABIB Regulation of muscle glycogen synthetase by metabolites Differential effects on the I and D forms *Biochemistry* 1968 *7* 56—66

PIRAS R. and R STANELONI *In vivo* regulation of rat muscle glycogen synthetase activity *Biochemistry* 1969 *8* 2153—2160

POSNER J B R STERN and E G KREBS Effects of electrical stimulation and epinephrine on muscle phosphorylase phosphorylase b kinase and adenosine 3 5 phosphate *J biol Chem* 1965 *240* 982—985

RAFAELSEN O J Glycogen content of rat diaphragm after intraperitoneal injection of insulin and other hormones *Acta physiol scand* 1964 *61* 314—322

SOVIK O The action of insulin on glycogen synthesis in rat diaphragm A comparative study of *in vitro* and *in vivo* effects *Acta physiol scand* 1966 *68* 246—254

STORK H and F H SCHMIDT Mitteilung uber eine enzymatische Schnellmethode zur Bestimmung des Blutzuckers in 5 μl Capillarblut ohne Enteiweissung und ohne Zentrifugation *Klin Wschr* 1968 *46* 789—790

THOMAS J A K K SCHLENDER and J LARNER A rapid filter paper assay for UDP glucose glycogen glucosyltransferase including an improved biosynthesis of UDP ^{14}C glucose *Anal Biochem* 1968 *25* 486—499

TORRES H N L R MARECHAL E BERNARD and E BELOCOPITOW Control of muscle glycogen phosphorylase activity by insulin *Biochim biophys Acta* (Amst) 1968 *156* 206—209

VILLAR PALASI C and J LARNER Insulin treatment and increased UDPG glycogen transglucosylase activity in muscle *Arch Biochem* 1961 *94* 436—442

VILLAR PALASI C M ROSELL PEREZ S HIZUKURI F HUIJING and J LARNER Muscle and liver UDP glucose α 1 4 glucan α-4 glucosyltran ferase (glycogen synthetase) In *Methods in Enzymology Vol 8* Eds Neufeld E and V Ginsberg Acad Press New York 1966 pp 374—384

WILLIAMS B J and S E MAYER Hormonal effects on glycogen metabolism in the rat heart *in situ Mol Pharmacol* 1966 *2* 454—464

WOOLF CH M *Principles of Biometry* D van Nostrand Company Inc Princeton New Jersey USA 1968

Acta physiol scand 1973 87 474—484

From the Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden

Effects of Ionic Concentration on Permeability Properties of Nodal Membrane in Myelinated Nerve Fibres of Xenopus laevis Potential Clamp Experiments

By

TOM BRISMAR

Received 11 July 1972

Abstract

BRISMAR T *Effects of ionic concentration on permeability properties of nodal membrane in myelinated nerve fibres of Xenopus laevis Potential clamp experiments* Acta physiol scand 1973 87 474—484

The specific sodium and potassium permeability mechanisms in the nodal membrane were analysed The current to potential relations were measured with various external concentrations of NaCl KCl and $CaCl_2$ P_N and P_K were calculated The measurements showed that the permeability to potential curve was shifted along the potential axis and in the same direction for changes in the uni univalent salt concentration as well as for changes in [Ca] The shift caused by changes in [Ca] was larger at low uni univalent salt concentrations and the shift caused by [K] and [Na] increased with decreased [Ca] It was assumed that the membrane has negative charges fixed to its outside surface The permeability shifts were approximately predicted by a charge density of -5.5 μC cm- A decrease in [Na] but not in [Ca] caused a decrease in P_K This behaviour was not predicted

Sodium and potassium are the main carriers of current in the squid nerve membrane (Hodgkin and Huxley 1952) and in the myelinated nerve fibre of *Xenopus* (Dodge and Frankenhaeuser 1959) Calcium and magnesium on the other hand seem to have their major effects by affecting the potential dependence of the sodium and potassium permeability changes (Frankenhaeuser 1957 Frankenhaeuser and Hodgkin 1957 Hille 1968 Brismar and Frankenhaeuser 1972) Some other excitable membranes deviate from this behaviour the barnacle giant muscle fibre can thus discharge impulses on the basis of current carried by calcium (Hagiwara Hayashi and Takahashi 1969) The conditions are however not even for the squid fibre and the frog nerve fibre so simple that other effects of these ions are excluded Thus it is well known that the delayed potassium current at large potential steps is smaller when sodium is replaced by choline chloride or sucrose (Hodgkin and Huxley 1952 Frankenhaeuser 1962 b) Further it is known that the potential threshold of the perfused squid giant axon is affected by the ionic concentration of the perfusate and

that the sodium inactivation curve is shifted along the potential axis by changes in ionic strength (Chandler Hodgkin and Meves 1965) The authors point out that these effects may be the consequence of negative charges fixed onto the inside surface of the excitable membrane Mozhayeva and Naumov (1970) found in a potential clamp analysis on the myelinated nerve fibre that calcium concentration ionic strength and pH affected the potential at which potassium permeability changes These experimental findings were interpreted on the basis of assumed fixed negative charges

The present investigation was made in order to analyse in further detail the effects on the ionic permeability changes of variations in the concentrations of sodium potassium and calcium The experimental measurements show that changes in the concentration of sodium and potassium chloride cause a shift of the permeability variables similar and of the same direction as that caused by calcium The shift caused by changes of [CaCl] was large in the region of low concentration of uni univalent salts and the shift caused by a change in the concentration of the uni univalent salts was correspondingly large at low concentrations of calcium while negligible at high [CaCl] These findings are interpreted on the basis of the assumption that (a) the specific ionic permeability depends on the potential at a critical site on the membrane (b) negative charges are fixed on the outside surface of the membrane (c) the free ions affect the potential caused by the fixed charges and (d) that the potential across the membrane and the potential caused by the fixed charges have a similar effect on the permeability determining mechanism

Methods

The membrane potential of single nodes of Ranvier in large nerve fibres from *Xenopus laevis* was changed in rectangular pulse steps The two-amplifier feed back technique used was as described by Dodge and Frankenhaeuser (1958) but modified for operational amplifiers (Brismar and Frankenhaeuser 1972)

The aim was to investigate the effect of changes in composition of the external solution on the sodium and potassium permeability properties of the nodal membrane All the measurements were made on fibres held at a polarization of sufficient strength to maintain sodium and potassium inactivation at a low level (i e $h_\infty \simeq 1$ and $k_\infty \simeq 1$) in order to avoid confusion through the effect of changes in the inactivation This was especially important whenever solutions with low [Ca] were used

The measurements of the steady state potassium permeability was made in the following way (a) the sodium equilibrium potential (E_{Na}) was determined (b) a conditioning step immediately followed by a test step to E_{Na} was applied (c) the instantaneous current at the beginning of the test step was measured the leak current was subtracted and the net current was taken as being proportional *to the potassium permeability at the end of* the conditioning step (Frankenhaeuser 1962 a) (d) this procedure was repeated with a suitable selection of conditioning steps to obtain a smooth steady state potassium permeability to potential curve (e) similar clamp runs were made with several different solutions (solutions of type A in Table I) applied to the node (No) under investigation

Measurements of the peak sodium permeability to potential curve were made in the ordinary way (a) test steps of suitable amplitudes were applied (b) the peak initial current was plotted against the potential during the test pulse (c) the leak current was subtracted (d) E_{Na} was determined (e) peak P_{Na} was calculated using the constant field equation and the measured I_{Na} Such determinations were made with node No in solutions of type B (see Table I)

Solutions The composition of the solutions used is given in Table I

Nomenclature Potentials are given as inside potential minus outside potential Outward currents are consequently given as positive

TABLE I *Composition of solutions Concentrations in mM When reference to a solution is given in the text then the solution is identified for type A solutions by [CaCl] and [NaCl] and for type B by [CaCl] and [KCl] Reference solutions Type A 2 0 mM CaCl and 112 mM NaCl type B 2 0 mM CaCl$_2$ and 90 0 mM KCl*

A Solutions with 2 5 mM KCl and 2 5 mM NaHCO$_3$

0 27 mM CaCl$_2$		2 0 mM CaCl$_2$		14 8 mM CaCl$_2$		109 mM CaCl	
NaCl	Sucrose	NaCl	Sucrose	NaCl	Sucrose	NaCl	Sucrose
115	–	112	–	93 0	–	72 0	–
55 0	120	52 0	120	33 0	120	–	–
25 0	180	22 0	180	2 8	180		
9 5	210	7 0	210				
2 1	225	–	224				

B Solutions with 25 0 mM NaCl and 2 5 mM NaHCO

0 27 mM NaCl		2 0 mM CaCl$_2$		14 8 mM CaCl	
KCl	Sucrose	KCl	Sucrose	KCl	Sucrose
90 0	–	90 0	–	70 0	–
30 0	120	30 0	120	–	140
–	180	–	180		
180	–	180	–		

Results

assium permeability

The isolated nerve fibre was mounted in the recording cell and the membrane potential was clamped A clamp run was made to determine the sodium equilibrium potential (E_{Na}) Conditioning steps each one immediately followed by a test step to E_N were applied The duration of the conditioning steps was 35 ms in order to get the potassium permeability turned on to its steady state value but not inactivated The potassium permeability at the end of the conditioning step was determined from the instantaneous current at the beginning of the test step and the constant field equation This determination was made after the experiment from photographic records of the currents

Similar runs and determinations were made with a large number of modifications of the external solution applied to node N_0 (solutions A in Table I) The number of solution changes in an experiment was large Repeated control runs were made with the solution A with 2 0 mM CaCl and 112 mM NaCl All measurements after the last well reversible control run were discarded from the material used

From the measurements the following findings were evident (1) the earlier finding (Frankenhaeuser 1962 b) that the maximum steady state P_K decreased somewhat in solutions with low [NaCl] was verified (see Fig 1) (2) the steady state P_K to membrane potential curve shifted along the potential axis in positive direction when [CaCl] was increased and in negative direction when [CaCl] was decreased

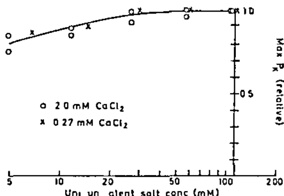


Fig 1 Steady state P_K at large positive potential steps (ordinates) plotted against uni univalent salt concentration (abscissae) P_K given relative to its value in ordinary Ringer solution Measurements as indicated in two [Ca] s Solutions of type A Table I 20 C

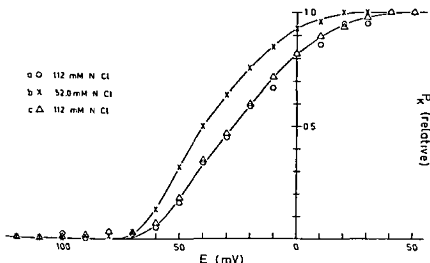


Fig 2 P_K (ordinates) as measured from instantaneous cu ent at a potential step to Ex from a conditioning step (abscissae) of 35 ms duration Solut ons of type A ith 2 0 mM CaCl Measurements made in alphabetical order Smooth curves drawn through experimental points Temp 20 C

(3) a similar shift was noted at one and the same [CaCl] when [NaCl] was varied (Fig 2) This shift was in positive direction with an increase in [NaCl] as the shift associated with changes in [CaCl]

In order to facilitate a comparison between the shift of the P_K curve caused by changes in [CaCl] and by changes in [NaCl] the following procedure was made The P_K potential curve was plotted for the runs in the various solutions The potential value was determined at which P_K had a value equal half the maximum value a large steps These values for the various test solutions minus the corresponding value for the reference solution were plotted (ordinates) against the uni univalent

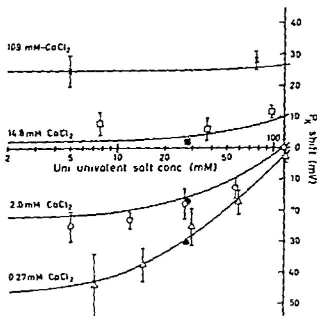


Fig 3 Plot of measurements of potential at which P_K equals half its maximum value in the test solution minus potential at which P_K equals half its value in the reference solution against uni univalent salt concentration. Measurements made as indicated with four different concentrations of calcium chloride Solutions of type A Table I Filled symbols indicate experiments where Ca was replaced by Mg Smooth lines drawn as solutions of equation (1) with a charge density of −5.5 μC cm Mean values ± S D

salt concentration (abscissae) Fig 3 is a plot of such mean values ± S D obtained from several experiments

The plot shows clearly that the shift caused by the changes in [CaCl] was larger at low concentrations of uni univalent salts while the shift associated with the change in uni univalent salt concentration was largest in solutions with low [CaCl]

This finding indicated that [Ca] and [Na] might affect the potassium permeability system in the same principal manner A common property of the two ionic species is that they have positive charge A trial was made to check how far some simple assumptions possibly could agree with the experimental findings It was assumed that (a) negative charges are fixed to the external surface of the membrane (b) the potential at the outside surface of the membrane is modified by the ionic composition of the external solution in a nonspecific manner (c) the potassium permeability is governed by the potential difference between the axis cylinder and the outside surface of the membrane

A quantitative treatment on these assumptions may be carried out on the basis of the mathematical description of either the ionic atmosphere in the vicinity of charges or the diffuse electrical double layer (Gouy 1910 Chapman 1913) This was used by Chandler *et al* (1965) for the treatment of experimental findings concerning the perfused squid giant nerve fibre A suitable approximation for a quantitative treatment is found in equation (1) which is a modification of equation (40) from Grahame (1947)

$$\sigma^2 = \frac{\varepsilon RT}{2\pi} \sum c_i \left(\exp\left\{ -\psi_{out} F/RT \right\} - 1 \right) \quad (1)$$

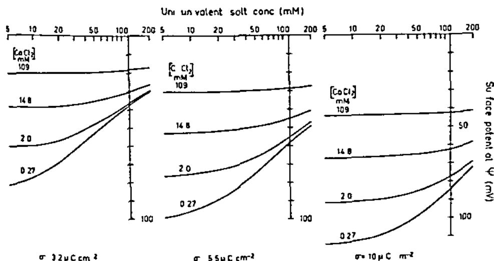


Fig 4 Surface potential ψ_0 as affected by ionic composition of solution Solutions of eqn (1) at charge densities $-3\ 2$ $-5\ 5$ and $-10\ \mu C\ cm^{-2}$ Ordinates surface potential minus potential of bulk solution Abscissae uni univalent salt concentration Solutions for four different calcium concentrations

σ = charge density (C cm)
ψ = surface potential minus potential of the bulk solution (V)
ε = dielectric constant of water in practical units (= 78 10^{1} C V^{1} cm^{1})
c_i = ith ionic concentration of solution (g ion cm^{3})
z_i = valence of ith ionic species
F = Faraday constant (= 96 500 C $equiv^{1}$)
R = Gas constant (= 8 3 J $mols^{1}$ $°K^{1}$)
T = Temperature (°K)

The charge density is required for a numerical solution of equation (1) This was unknown and no independent measurement of it was made The equation was therefore solved for a number of assumed charge densities Fig 4 gives three such solutions The charge density which gave a reasonable fit to the experimental measurements with the ordinary [NaCl] and different [Ca] s was assumed to be correct A family of curves as solutions of equation (1) for this charge density ($-5\ 5\ \mu C$ cm) and the used [Ca] s was drawn in Fig 3 It is seen that the whole family of curves agrees reasonably well with the experimental measurements

Sodium permeability

The effect of calcium on the potential dependent passive sodium transport system is known to be largely similar to that on the potassium system Experiments were therefore made to measure the effects of the concentration of the uni univalent on the sodium permeability mechanism The potassium concentration in t

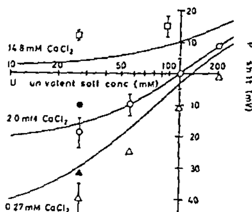


Fig 5

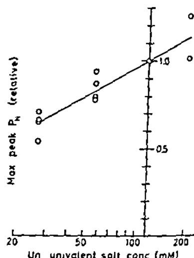


Fig 6

Fig 5 Plot of measurements of potential at which P_{Na} equals half its maximum value in the test solution minus potential at which P_{Na} equals half its value in reference solution, against uni univalent salt concentration Measurements made as indicated with three different concentrations of calcium chloride Solutions of type B Table I Filled symbols indicate experiments where Ca was replaced by Mg Smooth lines drawn as solutions of eqn (1) with a charge density of $-5.5\ \mu C\ cm$ Membrane potential held at $E = -110$ or $E = -120$ mV before test pulse Mean values $\pm$S D

Fig 6 Maximum peak constant field sodium permeability in units of peak P_{Na} in solution type B with 2 0 mM CaCl$_2$ and 90 0 mM KCl Membrane potential $E = -110$ or $E = -120$ mV before test pulse Abscissae uni univalent salt concentration Solutions of type B ble I

inder is large the potassium permeability system could therefore be investigated by observations of the outward potassium currents The internal sodium concentration is low and the outward sodium currents are correspondingly small The system was therefore investigated through measurements of the inward sodium currents The sodium chloride concentration was however reduced to 25 0 mM in order to make it possible to vary [KCl] over a relatively large range A further decrease of [Na] reduced the inward sodium currents too much for reliable measurements

Measurements were made of the peak sodium permeability with step pulses in the usual manner the peak initial current minus leak current was measured and plotted against the potential step the peak sodium permeability was calculated using the constant field equation These measurements were carried out with the various [CaCl] and [KCl] in the solutions applied to node N_0 (solutions B in Table I)

The major effect on the curves relating peak P_{Na} to membrane potential was in analogy with the findings on P_K that an increase in [CaCl] and in concentration of the uni univalent salt shifted the P_{Na} curve along the potential axis in positive direction To facilitate the comparison a plot was made of the potential at which peak P_{Na} was 50 % of the maximal peak P_{Na} in each solution minus the potential

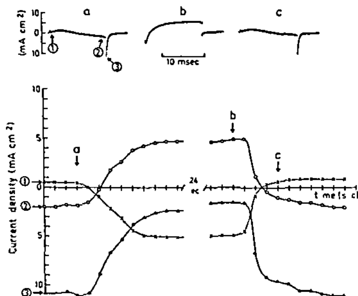


Fig 7 Time course of membrane currents (1) initial current (2) delayed current (3) instantaneous current at repolarization Potential before and after test step −110 mV during test step −44 mV Repetition rate 1 per s. Inset top samples of records marked a, b c Solution before first switch and after second switch type A with 2 0 mM-$CaCl_2$ and 90 0 mM KCl between first and second switch type A with 2 0 mM $CaCl_2$ and no KCl Temp 20 C

for the 50 % value in the reference solution (ordinates) against concentration (abscissae) This plot Fig 5 shows a striking similarity with the corresponding plot of the effects on the potassium mechanism Fig 3 The solutions of equation (1) for a charge density of −5 5 μC cm for the adequate concentrations of Ca are drawn as smooth lines in the Figure The general agreement between the experimental points and the smooth lines is reasonable

Additional experiments were made in which Ca^{++} was replaced by Mg^{++} These measurements are shown by filled symbols in Fig 3 and 5

The steady state potassium permeability decreased at very low uni univalent salt concentrations (Fig 1) Corresponding measurements were made on the sodium system. Fig 6 shows this dependence in the fibres investigated with type B solutions The limited specificity earlier described by Frankenhaeuser and Moore (1963) with a ratio of 20 1 for P_{Na} P_K of the channel for the initial current was observed as a change in the equilibrium potential This has been accounted for in Fig 6

The peak P_{Na} at large step pulses was somewhat larger in low [Ca] than in high This effect was smaller in high [Na] Further investigations are required on these points

Time course of ionic concentration effect

It was realized that the permeability changed rapidly when a new solution with another composition was applied. An experiment was arranged so that three different characteristic parts of the membrane current would change during a solution switch. A step pulse was applied from E = −110 mV to −44 mV and back to −110 mV. The first solution was solution type B with 2.0 mM $CaCl_2$ and 90.0 mM KCl while the second was type B with 2.0 mM $CaCl_2$ and no KCl. The pulse was applied with 1 s intervals and the membrane current was recorded. The characteristic features of the current in the first solution were (1) the sodium current was small because P_{Na} was not turned on at this step in this solution, (2) the delayed current was inward due to the high [K], (3) the potassium tail was large and inward. In the second solution again (1) the sodium permeability was turned on at the corresponding step, (2) the delayed current was outward and (3) the potassium tail was small.

Fig. 7 shows how these characteristic currents changed during the solution switches. It is seen that all three currents change simultaneously. The cell was not made for very rapid switches; the observed rates were very likely limited by the rate of the concentration change. The changes were more rapid at the second switch which is from a solution with high specific weight to one with low. The fibre is placed close to the upper surface in the cell and inflow as well as outflow are at top of the cell.

Discussion

The main finding in the present investigation was that the P_K to E curve and the P_{Na} to E curve shifted along the potential axis for changes in the uni-univalent salt concentration in the external solution in the same general manner as it shifted for changes in [Ca]. In these experiments the uni-univalent salts were exchanged by sucrose. The shift caused by changes in [Ca] was larger at low concentrations of uni-univalent salts than at high concentrations. The shift associated with changes in uni-univalent salt concentration was correspondingly larger at low [Ca]. The effects on P_K and on P_{Na} were very similar. In earlier investigations it was concluded that [Na] did not affect g_{Na} or P_{Na} (Hodgkin and Huxley 1952, Dodge and Frankenhaeuser 1959). The ionic strength was kept constant in these experiments and the effect would therefore not be present.

A trial was made to account for these effects on the basis of the effects of fixed negative charges on the external surface of the membrane. These charges will cause a potential difference between the surface and the bulk of the external solution and correspondingly between the outside surface of the membrane and the more internal parts of the membrane. The size of this surface potential is determined by the charge density, by the ionic composition and by the dielectric constants in the vicinity of the surface. Chandler *et al.* (1965) have explained by a charged layer the effect of ionic concentration on the inactivation of the sodium system. These authors give suitable equations for the treatment of concentration effects on the surface potential

caused by fixed charges The present treatment is closely related to the treatment just mentioned, but deals with divalent ions as well as with univalent ions A somewhat modified formal treatment (equation 1) was therefore used (see further Gouy 1910 Chapman 1913 Grahame 1947 Verwey and Overbeek 1948 Parsons 1954)

The present results are well consistent with the idea of fixed charges on the outside surface of the membrane and that the potential difference between the inside of the fibre and the outside surface is the potential difference which governs the permeability changes The charge density (−5 5 μC cm) corresponding to a distance of about 1 8 nm between single electronic charges is such that the potential decreases significantly between the individual charges The experimental findings give only an indication about the potential at the sites where potential determines the permeability These sites may be or may not be identical with the sites for the specific permeability channels If the fixed charges cause a potential step at the outside end of the sodium channels then the constant field rectification requires that a similar potential step affects the inside end of the nodal sodium channel (Frankenhaeuser 1960) The potential profile of the membrane surface may however be quite complicated It is further clear that the charge density used for the present calculations may deviate from a charge density obtained from electrophoretic measurements (see Curtis 1967)

I wish to thank Prof B Frankenhaeuser for fruitful discussions and helpful suggestions This work was supported by the Swedish Medical Research Council (Project No 14X 545) Karolinska Institutets fonder and Svenska Sallskapet for Medicinsk Forskning

References

BRISMAR T and B FRANKENHAEUSER The effect of calcium on the potassium permeability in the myelinated nerve fibre of *Xenopus laevis* *Acta physiol scand* 1972 *85* 237—241

CHANDLER W K A L HODGKIN and H MEVES The effect of changing the internal solution on sodium inactivation and related phenomena in giant axons *J Physiol* (Lond) 1965 *180* 821—836

CHAPMAN D L A contribution to the theory of electrocapillarity *Phil Mag* 1913 *25* 475—481

CURTIS A S G *The Cell Surface Its Molecular Role in Morphogenesis* Logos Press & Academic Press London 1967

DODGE F A and B FRANKENHAEUSER Membrane currents in isolated frog nerve fibre under voltage clamp conditions *J Physiol* (Lond) 1958 *143* 76—90

DODGE F A and B FRANKENHAEUSER Sodium currents in the myelinated nerve fibre of *Xenopus laevis* investigated with the voltage clamp technique *J Physiol* (Lond) 1959 *148* 188—200

FRANKENHAEUSER B The effect of calcium on the myelinated nerve fibre *J Physiol* (Lond) 1957 *137* 245—260

FRANKENHAEUSER B Sodium permeability in toad nerve and in squid nerve *J Physiol* (Lond) 1960 *152* 159—166

FRANKENHAEUSER B Instantaneous potassium currents in myelinated nerve fibres of *Xenopus laevis* *J Physiol* (Lond) 1962 a *160* 46—53

FRANKENHAEUSER B Potassium permeability in myelinated nerve fibres of *Xenopus laevis* *J Physiol* (Lond) 1962 b *160* 54—61

FRANKENHAEUSER B and A L HODGKIN The action of calcium on the electrical properties of squid axons *J Physiol* (Lond) 1957 *137* 218—244

FRANKENHAEUSER B and L E MOORE The specificity of the initial current in myelinated nerve fibres of *Xenopus laevis* Voltage clamp experiments *J Physiol* (Lond) 1963 *169* 438—444

Gouy G Sur la constitution de la charge électrique à la surface d un électrolyte *J physique* 1910 *9* 457—468
Grahame D C The electrical double layer and the theory of electrocapillarity *Chem Rev* 1947 *41* 441—501
Hagiwara S H Hayashi and K. Takahashi Calcium and potassium currents of the membrane of a barnacle muscle fibre in relation to the calcium spike *J Physiol* (Lond) 1969 *205* 115—129
Hille B Charges and potentials at the nerve surface Divalent ions and pH *J gen Physiol* 1968 *51* 221—236
Hodgkin A L and A F Huxley Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo J Physiol* (Lond) 1952 *116* 449—472
Mozhayeva G N and A P Naumov Effect of surface charge on the steady state potassium conductance of nodal membrane *Nature* (Lond) 1970 *228* 164—165
Parsons R Equilibrium properties of electrified interphases In *Modern Aspects of Electrochemistry* Editors J O M Bockris and B E Conway Butterworths Sci Publ London 1954 pp *103—179*
Verwey E J W and J Th G Overbeek *Theory of the Stability of Lyophobic Colloids* Elsevier Publ Comp Amsterdam 1948

Acta physiol scand 1973 87 485—497

From the Department of Physiology Gymnastik och Idrottshogskolan and the Department of Pediatrics Karolinska Institutet St Gorans Childrens Hospital Stockholm Sweden

Muscle Metabolism and Enzyme Activities after Training in Boys 11—13 Years Old

By

BENGT O ERIKSSON[1] PHILIP D GOLLNICK[1] and BENGT SALTIN

Received 11 July 1972

Abstract

ERIKSSON B O P D GOLLNICK and B SALTIN *Muscle metabolism and enzyme activities after training in boys 11—13 years old* Acta physiol scand 1973 87 485—497

The effect of training on the skeletal muscle metabolism of 11 to 13 year-old boys was examined In one experiment changes in blood lactate and muscle lactate CP ATP and glycogen were determined at rest and following exercise before and after 4 months of training The concentrations of glycogen CP and ATP at rest were higher ($P < 0.01$) following training Blood and muscle lactate were 23 and 56 % higher after maximal work following training A greater reduction in muscle glycogen occurred during maximal work after training but the pattern for ATP and CP depletion was unchanged In a second experiment boys trained by pedalling a bicycle ergometer an average of 30 min 3 times a week for 6 weeks Biopsy samples of the vastus lateralis were examined for oxidative (succinate dehydrogenase) and anaerobic (phosphofructokinase) capacity before and after training The fiber composition and relative oxidative capacity in the fibers as determined histochemically Succinate dehydrogenase and phosphofructokinase activities increased 30 and 83 % respectively following training Fiber distribution was unchanged by training but the oxidative capacity of both fiber types appeared to increase

Blood lactate concentration at submaximal and maximal exercise is lower in sedentary young boys as compared to both untrained and trained adults (Åstrand 1952) The blood lactate values in boys are related to a lower production of lactate as judged by measurements of muscle lactate concentrations and oxygen deficits after the onset of work (Eriksson Karlsson and Saltin 1971) If young boys go through a conditioning program which in adults results in a lowered lactate accumulation at submaximal work (Ekblom *et al* 1968 Karlsson *et al* 1972) the question arises as to whether such training will result in a further lowering of the blood lactate concentration at submaximal work To study this problem eight 11 to 13 year-old boys were examined at rest and during exercise before and after completing a 4 month training program

[1] Current addresses P D G Department of Physical Education for Men, Washington State University Pullman Washington USA B O E Department of Pediatrics University Gothenburg SWEDEN

Based on in vitro studies it has been suggested that the concentration of the enzyme phosphofructokinase (PFK) is rate limiting in glycolysis (Danforth and Lyon 1964) Therefore it was also thought of interest to determine PFK activity in the skeletal muscle of young boys Moreover since muscle fiber composition glycogen content as well as the oxidative potential of the fibers may influence glycolysis and lactate production these measurements were included in an additional training study where 5 boys trained on a bicycle for 6 weeks

Subjects and Methods

Thirteen 11 to 13 year-old boys who attended the same elmentary school participated in the 2 studies Some of these boys belonged to a swimming club but none were engaged in any regular hard physical training Eight boys participated in the 4 month training program (Series I) and 5 boys in the 6-week training program (Series II) The physical characteristics of the subjects are summarized in Table I Medical examinations of the boys before during and after the training period did not reveal any disorders

Exercise tests were performed on an electrically braked cycle ergometer (Elema) at a pedal rate of 60 rpm. Heart rates were obtained from ECG tracings Expired air was collected in Douglas bags and its volume measured in a Tissot spirometer Analyses of the expired air were made with a modified Haldane technique Blood lactate was determined with an enzymatic method (Scholz *et al* 1959) on samples obtained from the fingertip

Samples of the vastus lateralis muscle were obtained with the needle biopsy technique (Bergstrom 1962) In series I they were immediately frozen in liquid nitrogen and stored at −80 C until analyzed Analyses for glycogen pyruvate lactate ATP (adenosine triphosphate) CP (creatine phosphate) glucose and glucose-6 phosphate (G-6-P) were made according to the methods described by Karlsson (1971) Metabolite concentrations are expressed as wet weight values The water content of the muscle samples at rest averaged 75 7 % and increased to 77 5 and 78 4 % after submaximal and maximal exercise respectively

In the second series the muscle samples were divided into 3 parts One portion of the sample was immediately frozen in liquid nitrogen and stored in dry ice for subsequent histochemical analysis The remaining 2 parts were weighed and used to determine succinate dehydrogenase SDH and PFK activities at 25 C with the methods of Cooperstein *et al* (1950) and Shonk and Boyer (1964) respectively

The samples frozen for histochemical analysis were examined under a dissecting microscope at −25 C to determine fiber orientation They were then cooled to the temperature of liquid nitrogen and placed onto specimen holders in OCT embedding medium (Ames Tissue Tek) at near 0 C and immediately immersed in liquid nitrogen Serial sections 10 μm thick were cut in a cryostat at −20 C and mounted on cover glasses for staining Myosin adenosine triphosphatase ATPase) and reduced diphosphopyridine nucleotide-diaphorase (DPNH-diaphorase) activities were estimated with the methods of Padykula and Herman (1955) and Novikoff and co-workers (1961) respectively The distribution of glycogen in one serial section (16 μm thick) was estimated from the periodic acid Schiff's (PAS) reaction (Pearse 1961) Photographs of the slides were taken and 20 × 25 cm black and white prints made for the purpose of classifying fibers and estimating the distribution of oxidative capacity in the different fibers

Ribonucleic acid (RNA) was determined in the muscle samples with the modified Schmidt—Thannhauser method described by Munro and Fleck (1966) Yeast RNA was used as the standard.

Procedures

Series I was undertaken from December 1970 to May 1971 The training took place from the middle of January to the middle of May The conditioning program was performed 3 times a week Each of the 34 training sessions lasted 60 min They usually included 5—10 min of calisthenics on the floor 15—25 min of interval running and basketball and soccer In addition to this weekly training all of the boys went to a training camp for a week where they also participated in cross country skiing twice a day The boys were studied before and after the training program. Biopsies were taken at rest and after 4 min of work at 500 and 750 kpm × min⁻¹ This last work load was maximal for 2 of the boys before the training

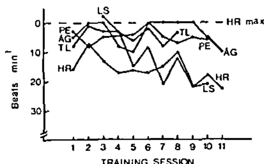


Fig 1 Individual values for heart rate during each training session (Series II) expressed in beats × min⁻¹ below the subject's highest observed heart rate in any maximal test

period started but for none of them after the training period In those cases where 750 kpm × min⁻¹ was not a maximal work load a heavier work load was also performed A 15 min rest period was interspaced between each work period The biopsies at rest were taken with the boys lying down and during work while they were seated on the bicycle In both situations the samples were frozen within 3—5 s Blood from the fingertip was taken at the same time as the biopsy samples

Heart rate was recorded for 15 s of each min of work Expired air for the determination of oxygen uptake (V_{O_2}) was collected continuously throughout each work period One collection was made during the first 3 min and another from the 3rd min to the end of the exercise During submaximal exercise the second collection of expired air was always between the 3rd and 4th min A 4 min work period was used to make the results of the present study comparable with studies on adults (Knuttgen and Saltin 1972 a b)

During submaximal work the V_{O_2} observed during the final collection period was of the same order of magnitude as that observed in these boys when the exercise continued for 6 to 8 min This was true both before and after training Thus V_{O_2} during the 3rd to 4th min of the exercise can be used to calculate the oxygen deficit for the submaximal work This was done by subtracting the V_{O_2} obtained during the first 3 min of exercise from 3 times the V_{O_2} observed between the 3rd and 4th min of work One factor complicating this procedure was the fact that boys have a higher mechanical efficiency than adults Therefore it could not always be stated if the observed V_{O_2} between the 3rd and 4th min of exercise was the normal value for the boys at steady state As all the boys in the present study were exercised on many different occasions with work periods of 6—10 min duration these V_{O_2} values could be used for this evaluation (Eriksson and Koch 1972) At maximal exercise oxygen deficit was calculated in the manner suggested by Karlsson (1971)

In Series I testicular volume was determined by the method described by Prader (1966) During the training the volume only increased from 5 4 to 8 7 cm³ ($P < 0.05$) This latter values is well below what is considered an indication of sexual maturity and testosterone production (Tanner 1962) Therefore these measurements were not included in Series II

All biopsy samples in Series II were taken at rest This was done before and after 2 and 6 weeks of physical training The training program in Series II consisted of pedalling a bicycle 3 times a week for a total of at least 20 min (range 20—50 min mean 29 8) for each training session Heart rate response during the training program was counted and the mean value for each boy during the training session are given in Fig 1

Mean standard deviations and standard errors were calculated using standard techniques (Snedecor 1961) Student's t test was applied using the method for paired observations The levels of significance as indicated in the tables are as follows * = $p < 0.05$ ** = $p < 0.01$ and *** = $p < 0.001$

Results

Series I

The boys in this series increased their max V_{O_2} with 0 28 l/min or 15 % (Table I) This was more than expected from the growth that took place during the training period (von Dobeln and Eriksson 1972)

TABLE Ia Anthropological and physiological data in series I before (B) and after (A) training

		Series I		
		Mean ± S E	Range	significance
Age years	B	11 5 ± 0 24	11 0 – 13 0	
	A	12 1 ± 0 24	11 6 – 13 6	
Height m	B	1 492 ± 0 032	1 375 – 1 614	
	A	1 525 ± 0 035	1 409 – 1 668	***
Weight kg	B	44 7 ± 2 69	35 0 – 57 3	
	A	45 4 ± 2 76	33 1 – 55 0	n s
Testicular volume cm³	B	5 3 ± 1 2	2 0 – 11 0	
	A	8 7 ± 2 1	2 0 – 17 5	*
Maximal oxygen uptake l min⁻¹	B	1 87 ± 0 09	1 52 – 2 43	
	A	2 15 ± 0 12	1 73 – 2 85	**
Maximal heartrate beats min⁻¹	B	196 ± 2 0	186 – 200	
	A	193 ± 2 2	183 – 200	n s

Mean values for the metabolites measured are given in Table II The ATP concentration of muscle at rest was 4 3 and 4 8 mmol × kg⁻¹ ($P < 0\ 01$) before and after training respectively A small decrease in ATP occurred during exercise At maximal work this amounted to 0 9 mmol × kg⁻¹ The resting level of CP was also higher ($P < 0\ 01$) after as compared to before training the actual values being 20 2 and 14 5 mmol × kg⁻¹ respectively A marked drop in the CP concentration of muscle

Ib Anthropological and physiological data in series II before (B) after 2 weeks training (A2) and after 6 weeks training (A6)

		Series II	
		Mean ± S E	Range
Age years	B	11 2 ± 0 1	10 9 – 11 5
	A2		
	A6	11 3 ± 0 1	11 0 – 11 6
Height m	B	1 457 ± 0 027	1 385 – 1 510
	A2	1 454 ± 0 024	1 385 – 1 505
	A6	1 461 ± 0 022	1 405 – 1 510
Weight kg	B	36 7 ± 2 10	30 6 – 41 5
	A2	36 3 ± 1 93	30 6 – 40 4
	A6	36 6 ± 2 05	30 9 – 41 0
Maximal oxygen uptake l min⁻¹	B	1 93 ± 0 09	1 72 – 2 15
	A2	1 97 ± 0 10	1 72 – 2 24
	A6	2 05 ± 0 09	1 82 – 2 26
Maximal heart rate beats min⁻¹	B	204 ± 2 4	200 – 213
	A2	207 ± 2 1	201 – 213
	A6	200 ± 3 3	192 – 210

Only maximal oxygen uptake showed a significant increase ($P < 0\ 01$) after 6 weeks of training

TABLE II Oxygen deficit during exercise and blood lactate and muscle metabolite concentrations at rest and during exercise before (B) and after (A) 4 months of training in 11—13 years old boys Numbers of subjects = 8 except when indicated Levels of significance * = p < 0 05 ** = p < 0 01 *** = p < 0 001 1) n = 6 2) n = 7

Condition kpm × min⁻¹		Oxygen deficit l		Blood lactate mmol × l		ATP mmol × kg⁻¹		CP mmol × kg⁻¹		Glycogen glucose units mmol × kg⁻		Muscle lactate mmol × kg⁻¹		Glucose mmol kg		Glucose 6-phosphate mmol × kg⁻¹	
		B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
Rest	M			0 8	1 2	4 3	4 8	14 5	20 2	54	71	1 0	1 5	0 5	0 6	0 2	0 4
	S E			0 1	0 1	0 1	0 1	0 8	0 8	3	3	0 2	0 2	0 1	0 1	0 1	0 1
	Sign			*		**		**		*		n s		n s			
500	M	0 58	0 69	2 3	2 1	4 3	4 8	9 3	14 7	51	58	3 0	3 3	0 9	0 9	0 6	0 9
	S E	0 09	0 22	0 3	0 3	0 3	0 3	1 0	1 9	5	3	0 5	0 7	0 1	0 2	0 2	0 1
	Sign	n s		n.s		n.s		*		n.s		n s		n s		n s	
750[1]	M	1 04	1 08	3 7	3 0	4 2	4 1	6 0	9 6	44	47	6 7	5 6	1 3	1 4	1 2	1 0
	S E	0 10	0 10	0 6	0 7	0 2	0 3	0 8	1 2	8	6	1 2	1 8	0 2	0 2	0 2	0 2
	Sign	n.s		*		n s		n s		n.s		n s		n s		n s	
Maximal exercise (B = 850 A = 1014)	M	1 48	1 64	4 7	5 9	4 0	3 9	4 4	6 0	33	34	8 8	13 7	1 7	1 9	0 9	1 3
	S E	0 13	0 08	0 6	0 7	0 2	0 2	0 8	0 9	5	5	1 4	1 2	0 2	0 2	0 1	0 2
	Sign	*		**		n s		n s		n s		*		n s		n s	

occurred during exercise This change was closely related to the severity of the work with values around 5 mmol × kg⁻¹ being found at exhaustion The pattern for CP as well as ATP depletion was similar both before and after training (Fig 2)

Mean muscle glycogen concentrations were 53 9 and 71 0 mmol of glucose units × kg⁻¹ respectively before and after training ($P < 0\ 01$) A gradual depletion in glycogen was seen with each work level There was a tendency for a larger glycogen depletion after as compared with before training This difference was significant at maximal work However this is explained at least partly by the fact that the subjects performed a greater amount of work after traning G 6 P and glucose concentrations at rest were 0 2 and 0 5 mmol × kg⁻¹ respectively The concentration of both these compounds increased during exercise to 1 3 and 1 7 mmol × kg⁻¹ respectively at exhaustion Muscle glucose at rest and its pattern of change during exercise were not altered by training G 6 P levels were also similar before and after training but tended to be slightly higher after training both at rest and during work

Muscle lactate concentration at rest averaged 1 0 mmol × kg⁻¹ During exercise this increased to 3 0 and 6 7 mmol × kg⁻¹ at 500 and 750 kpm respectively with a concentration of 8 8 mmol × kg⁻¹ being reached after maximal exercise Similar values were found both at rest and at 500 and 750 kpm × min⁻¹ after training (Fig 3) A muscle lactate concentration of 13 7 mmol × kg⁻¹ was attained ($P < 0\ 05$) following maximal exercise after training Blood lactate concentrations at exhaustion

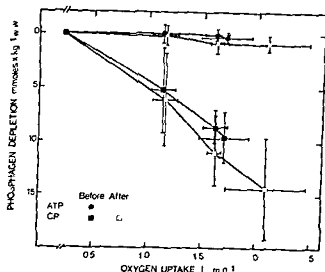


Fig 2 Mean values ± S D for ATP and CP depletion before and after training in relation to the oxygen uptake (Series I)

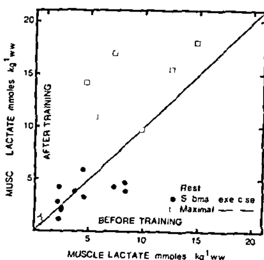


Fig 3 Individual values for the lactate concentration in the muscle at rest submaximal (500 and 750 kpm × min⁻¹) and maximal exercise before and after training for the boys in Series I

during submaximal and maximal work were lower but paralleled those of muscle lactate (Fig 4) The mean values at exhaustion were 4 8 and 5 9 mmol × l⁻¹ before and after training ($P < 0\ 01$) respectively

Prior to training the oxygen deficits were 0 58 1 02 and 1 48 l at 500 750 kpm × min⁻¹ and exhaustion respectively Slightly higher values were obtained at the same absolute work levels after training At maximal exercise the mean value was also increased and averaged 1 64 l ($P < 0\ 05$)

Series II

The boys in this part of the study had a mean max V_{O_2} of 1 93 l × min⁻¹ at the start of training and it gradually increased reaching 1 97 and 2 05 l × min⁻¹ after 2 and 6

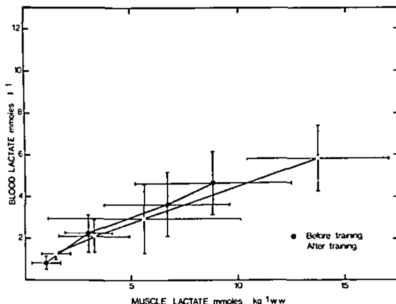


Fig 4 A comparison between the mean values ± S.D for muscle lactate and blood lactate concentration before and after training (Series 1)

weeks of training respectively (Table I) All boys exhibited an increase in max V_{O_2} The average *percent increase in max V_O was only 8 % but as almost no growth* took place during the training period the observed increase in V_{O_2} must be related to the physical conditioning Height increased an average of 0 004 m and weight de creased 0 1 kg Mean values for the local changes that took place in the skeletal

TABLE III Succinate dehydrogenase (SDH) and phosphofructokinase (PFK) activities ribonucleic acid (RNA) and slow twitch fibers (ST) in 5 11 year-old boys (Series II) before (B) and after 2 (A2) and 6 (A6) weeks of training

		Mean ± S E	Range	Difference	Significance
SDH µmol × (g × min)⁻¹	B	5 43 ± 0 41	3 85 – 6 27	A2 – B	n s
	A2	5 84 ± 0 57	4 57 – 7 40	A6 – B	
	A6	7 01 ± 0 42	6 16 – 8 06	A6 – A2	n s
PFK µmol × (g × min)⁻	B	8 42 ± 1 46	6 05 – 19 76	A2 – B	n s
	A2	12 46 ± 1 09	9 68 – 14 72	A6—B	*
	A6	15 41 ± 1 62	10 21 – 18 78	A6 – A2	
RNA mg × g⁻	B	1 74 ± 0 17	1 41 – 2 32	A2 – B	n.s
	A2	1 70 ± 0 07	1 55 – 1 93	A6 – B	n s
	A6	1 72 ± 0 11	1 15 – 1 86	A6 – A2	n s
ST	B	—	—	—	—
	A2	54 8 ± 3 4	45 1 – 60 3		
	A6	48 9 ± 3 4	40 2 – 54 6	A6 – A2	n s

The percentage of fast twitch fibers (FT) can be obtained by subtracting the percent of slow twitch fibers (ST) from 100

Levels of significance are for intra individual differences before and after training

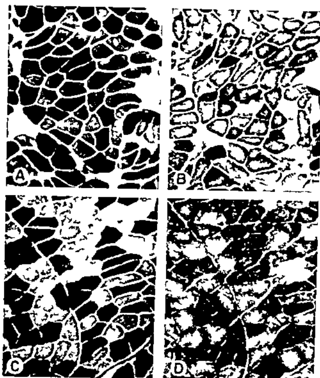


Fig 5 Serial sections (X130) from the vastus lateralis muscle before (A and B) and after (C and D) training A and C are sections showing fibers with high (dark) and low (light) myosin ATPase activity Sections B and D have been stained for oxidative capacity with the DPNH diaphorase method Both fiber types appeared to be more oxidative after training

cle are given in Table III RNA averaged 1 74 mg×g $^{-1}$ at the start of training and no significant change was observed as a consequence of training This was expected since increases in RNA concentration have only been observed during periods of extremely fast growth of muscle (Meerson 1969)

The muscle samples contained 54 9 % fibers with low myosin ATPase activity (slow twitch fibers Gollnick *et al* 1972) shortly after the start of training as compared with a mean of 49 2 % at the end of training In studies on adults where multiple biopsies have been taken a 5 % (S D) variation between samples is a common finding (Gollnick *et al* 1972) Thus the 5—6 % difference observed in the present study has no biological significance From the PAS staining it appeared that glycogen was stored equally in both fiber types regardless of the training indicating an enhanced storage of glycogen in the muscle The PFK activity before training was 8 4 μmol×(g×min) $^{-1}$ This activity was less than 50 % of that usually observed in adults (Gollnick *et al* 1972) PFK activity had risen to 12 5 and 15 4 μmol×(g×min) $^{-1}$ after 2 and 6 weeks of training respectively SDH activity was 5 4 μmol×(g×min) $^{-1}$ which is about 20 % higher than that observed in sedentary adults (Gollnick *et al* 1972) After 2 weeks of training the value was only increased to 5 8 μmol×(g×min) $^{-1}$ This small increase can be partly explained by the finding of a lowered SDH activity for one subject who had a rubeola infection and who consequently was unable to train during the first 2 weeks of the study His max V_{O_2} was

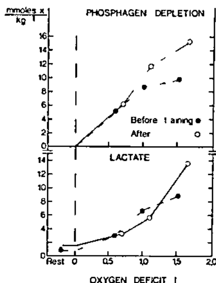


Fig 6 Mean values for lactate and phosphagen depletion in the muscle in relation to the oxygen deficit after four min of work (Series I)

also unchanged between these occasions After 6 weeks of training all of the boys exhibited higher SDH activities with the mean value being 7 0 μmol$\times$(g$\times$min)$^{-1}$ (P$<$0 05) DPNH diaphorase staining which is an indication of oxidative capacity and mitochondrial density was also more pronounced in the muscle sections at the end of the training program as compared to before training (Fig 5) The increase in oxidative capacity appeared to have taken place both in fibers with low and high myosin ATPase activity

Discussion

In previous studies by Karlsson (1971) and Knuttgen and Saltin (1972 a) it was demonstrated that the lactate level of muscle did not increase until the oxygen deficit surpassed 1 5 l Above this limit lactate accumulated in relation to the increase in oxygen deficit Moreover a maximal phosphagen depletion of approximately 12—14 mmol$\times$kg^{-1} was observed at an oxygen deficit of 3 l The same general relationship was observed in the boys of this study (Fig 6) but lactate appeared in exercising muscle at an oxygen deficit of 0 5 l and an almost maximal phosphagen depletion at 1 0 l To a large extent the observed differences in the absolute magnitude of oxygen deficit in the present study as compared with studies in adults most likely can be explained by the differences in physical size and muscle mass that exists between adult men and boys However the order of magnitude of the difference in oxygen deficit is larger than the difference in body size This speaks in favour of the probability that other factors are also brought into play in regulating the anaerobic energy yield in children during exercise Another sign of this may be that the boys exhibited much lower oxygen deficits than adults not only when the exercise

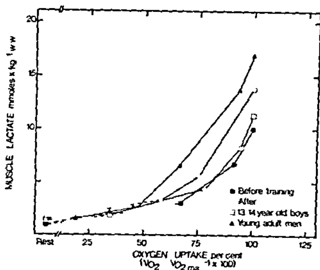


Fig 7 Mean muscle lactate concentration in relation to the oxygen uptake expressed in per cent of the individual max V_{O_2} (Series I) Included in the figure is data from 13—14 year-old boys (Eriksson Karlsson and Saltin 1971) and on adult men (Karlsson Diamant Saltin 1971 Knuttgen and Saltin 1972 a b)

pressed in absolute terms but also when related to the relative work level (*cf* Knuttgen and Saltin 1972 a)

The lower blood lactate of the 11 to 13 year old boys as compared to adults both at rest and after submaximal work agrees with several earlier reports (Åstrand 1952 Eriksson Karlsson and Saltin 1971) The muscle lactate concentrations in the boys following training were higher at all relative loads than the pre training values (Fig 7) This could have resulted from the 6 month period of maturation that occurred during the training program However this possibility seems remote since muscle lactate concentrations of untrained 13 to 14 year olds boys during submaximal work are similar to the pre training levels observed in this study (Eriksson *et al* 1971) The higher PFK activity in the skeletal muscle of the boys in the second training experiment strongly suggests that training increases the glycolytic potential of skeletal muscle of young boys In addition glycogen depletion during exhaustive exercise was greater after as compared to before training This also points to an increased glycolytic capacity in the skeletal muscle of the boys following training This effect of training on the glycolytic capacity of skeletal muscle is in contrast to a lack of any significant difference in PFK activity of training as compared to untrained men (Gollnick *et al* 1972) On the other hand the PFK activity in the muscle of adult men is 2 5 to 3 fold higher than the values observed in the present group of boys when untrained

Although muscle lactate concentrations were higher during exercise following training they were not closely related to the differences in the rate of glycogen depletion This may be explained in part by the fact that at a given relative work load more work was done after than before training since the max V_{O_2} had increased during training On the basis of the approximately 30 % increase in skeletal muscle SDH activity following training it could be postulated that the smaller lactate

production per unit of glycogen broken down after training resulted from a greater oxidative capacity of the muscle On the other hand the increase in oxidative capacity did not have a glycogen sparing effect or produce a lower muscle lactate at submaximal work as such a change has been suggest to produce (Holloszy 1967 Mole, Oscai and Holloszy 1971) These findings suggest that in the present experiment the increased glycolytic and oxidative capacity of skeletal muscle induced by training combined to produce a greater lactate production and work capacity

In contrast to muscle blood lactate tended to be lower at heavier work loads after training This may have resulted from a greater extraction of lactate by other tissues or from a different rate of production and utilization by the different fiber types in the working muscle The increased oxidative capacity of the fibers with low myosin ATPase activity would allow for a greater lactate utilization by these fibers during exercise

The magnitude of the increase in skeletal muscle SDH activity following training was similar to that reported by Varnauskas *et al* (1970) and Morgan and co-workers (1971) for adult men The increased oxidative potential was also observed in the histochemical staining for DPNH diaphorase activity with both fiber types appearing to have higher activities after training (Fig 5) This increased oxidative potential of both fiber types is consistent with earlier differences observed between trained and untrained men (Gollnick *et al* 1972) as well as the effect of training on rat skeletal muscle (Baldwin *et al* 1972)

Although SDH activity was about 30 % higher in the muscles of the boys of the second series max V_{O_2} increased less than 10 % This suggests that the oxidative capacity of the muscle may not limit total body oxygen consumption On the other hand the change in the SDH activity of the vastus lateralis following training with bicycle exercise may not reflect changes in all muscle groups since this muscle is probably used more extensively in bicycle work as compared to many other muscles of the body

No consistent pattern of a selective glycogen storage in either fiber type was discernible either before or after training from the PAS staining This agrees with an earlier report (Gollnick *et al* 1972) where large differences in the glycogen content of human muscle existed between trained and sedentary men

Muscle fibers have been classified on the basis of oxidative potential as measured histochemically by DPNH diaphorase or SDH activity or staining with sudan black (Buchthal and Schmalbruch 1970 Barnard Edgerton and Peter 1971 Faulkner *et al* 1971 Morgan *et al* 1971 Baldwin *et al* 1972) The increase in DPNH diaphorase activity of the muscle fibers after training as observed in the present study would support these previous findings However we have used myosin ATPase activity to classify fibers In this study a slightly higher percentage of fibers with high myosin ATPase activity was found in the post training muscle samples From these data it is tempting to suggest that training produced a change in fiber types in these subjects However such a conclusion is not warranted from the few subjects and small samples studied

The higher ATP concentration in skeletal muscle after training observed in this study is consistent with several earlier reports (Karlsson Diamant and Saltin 1971 Karlsson *et al* 1972) This can probably be attributed to the increase in mitochondrial concentration as indicated by the increased SDH activity and the histochemical staining for DPNH-diaphorase activity The increase in CP is more difficult to explain as it has not been seen in previous training studies on men However increases in CP have been reported in animal muscle following training (Ferdman and Feinschmidt 1929)

From the results of this study it is quite clear that marked local adaptations take place in skeletal muscle of boys following training Most changes are similar to those observed in adults and in animals but some differences in the training response of boys also seem to exist Moreover the observation of an increased glycolytic capacity as a consequence of training during growth should be investigated further

This study was supported by grants from Semper Nutrition Foundation and Swedish Medical Research Council (project No 40X 2203)

References

ÅSTRAND P O *Experimental studies of physical working capacity in relation to age and sex* Munksgaard Copenhagen 1952

BALDWIN K M G H KLINKERFUSS R L TERJUNG P A MOLE and J O HOLLOSZY Respiratory capacity of white red and intermediate muscle adaptative response to exercise *Amer J Physiol* 1972 *222* 373—378

BARNARD R J V R EDGERTON and J B PETER Effect of exercise on skeletal muscle I Biochemical and histochemical properties *J appl Physiol* 1970 *28* 762—766

BERGSTROM J Muscle electrolytes in man *Scand J clin Lab Invest* 1962 *14* Suppl 68

BUCHTAL F and H SCHMALBRUCH Contraction times and fiber types in intact human muscle *Acta physiol scand* 1970 *79* 435—452

COOPERSTEIN S J A LAZAROW and N J KURFESS A microspectrophotometric method for the determination of succinic dehydrogenase *J biol Chem* 1950 *186* 129—139

DANFORTH W H and J B LYON JR Glycogenolysis during tetanic contraction of isolated mouse muscles in the presence and absence of phosphorylase a *J biol Chem* 1964 *238* 4047—4052

VON DOBELN W and B O ERIKSSON Physical training maximal oxygen uptake and dimensions of the oxygen transporting and metabolising organs in boys 11—13 years of age. *Acta paediat scand* 1972 *61* 653—660

EKBLOM B P O ÅSTRAND B SALTIN J STENBERG and B WALLSTROM Effect or training on circulatory response to exercise *J appl Physiol* 1968 *24* 518—528

ERIKSSON B O J KARLSSON and B SALTIN Muscle metabolites during exercise in pubertal boys *Acta paediat scand* 1971 Suppl 217 57—63

ERIKSSON B O and G KOCH Effect of physical training on hemodynamic response during submaximal and maximal exercise in 11—13 year old boys *Acta physiol scand* 1973 *87* 27—39

FAULKNER J A L O MAXWELL D A BROOK and D A LIEBERMAN Adaptation of guinea pig plantaris muscle fibers to endurance training *Amer J Pysiol* 1971 *221* 291—297

FERDMAN D and O FEINSCHMIDT Über den Einfluss des Trainierens des Muskels auf seinen Gehalt an Phosphorverbindungen *Hoppe Seylers Z physiol Chem* 1929 *183* 216—268

GOLLNICK P D R B ARMSTRONG C W SAUBERT IV K PIEHL and B SALTIN Enzyme activity and fiber composition in skeletal muscle of untrained and trained men *J appl Physiol* In press

HOLLOSZY J O Biochemical adaptations in muscle Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle *J biol Chem* 1967 *242* 2278—2282

KARLSSON J Lactate and phosphagen concentrations in working muscle of man *Acta physiol scand* 1971 Suppl 358

KARLSSON J B DIAMANT and B SALTIN Muscle metabolites during submaximal and maximal exercise in man *Scand J clin Lab Invest* 1971 *6* 358—394

KARLSSON J L O NORDESJO L JORFELDT and B SALTIN Muscle lactate ATP and CP levels during exercise after physical training in man *J appl Physiol* 1972 *33* 194—203

KNUTTGEN H and B SALTIN Muscle metabolites and oxygen consumption in short term submaximal exercise in man *J appl Physiol* 1972 a *32* 690—694

KNUTTGEN H and B SALTIN Oxygen uptake muscle high energy phosphates and lactate in exercise under acute hypoxic conditions in man *Acta physiol scand* 1973b *87* 368—376

MEERSON F Z The myocardium in hyperfunction hypertrophy and heart failure *Circulat Res* 1969 *25* Suppl 2

MOLE P A L B OSCAI and J O HOLLOSZY Adaptation of muscle to exercise Increase in levels of palmityl CoA synthetase carnitine palmityltransferase and palmityl CoA dehydrogenase and in the capacity to oxidize fatty acids *J clin Invest* 1971 *50* 2323—2330

MORGAN T E L A COBB F A SHORT R ROSS and D R GUNN Effects of long term exercise on human muscle mitochondria In *Muscle Metabolism During Exercise* ed B Pernow and B Saltin Plenum Press New York 1971 87—95

MUNRO H N and A FLECK The determination of nucleic acids *Methods biochem Anal* 1966 *14* 113—176

NOVIKOFF A B W SHIN and J DUCKER Mitochondrial localization of oxidation enzymes Staining results with two tetrazolium salts *J biophys biochem Cytol* 1961 *9* 47—61

PADYKULA H A and E HERMAN The specificity of the histochemical method of adenosine triphosphatase *J Histochem Cytochem* 1955 *3* 170—195

PEARSE A G E *Histochemistry—Theoretical and Applied* Little Brown and Company Boston 1961 Appendix 9 832

PRADER A Testicular size Assessment and clinical importance *Triangle* 1966 *7* 240—243

SCHOLZ R H SCHMITZ T BUECHER and J O LAMPEN Über die Wirkung von Nystatin auf Backerhafe *Biochem Z* 1959 *331* 71—86

SCHONK C E and G E BOXER Enzyme patterns in human tissue I Methods for the determination of glycolytic enzymes *Cancer Res* 1964 *24* 709—724

SNEDECOR G S *Statistical methods* Iowa State University Press Ames Iowa 1961

TANNER J H *Growth at adolescence* Blackwell Scientific Publications Oxford 2 ed 1962

VARNAUSKAS E P BJORNTORP M FAHLEN I PREROVSKY and J STENBERG Effects of physical training on exercise blood flow and enzymatic activity in skeletal muscle *Cardiovascular Res* 1970 *4* 418—422

Acta physiol scand 1973 87 498—506

From the Institute of Aviation Medicine Flight Test Centre Malmslatt Sweden and the U S Army Research Institute of Environmental Medicine Natick Massachusetts

Effects of Alcohol, Hot Drinks, or Smoking on Hand and Foot Heat Loss

By

RALPH F GOLDMAN, RUSSEL W NEWMAN and OVE WILSON

Received 11 July 1972

Abstract

GOLDMAN R F R W NEWMAN and O WILSON *Effects of alcohol hot drinks or smoking on hand and foot heat loss* Acta physiol scand 1973 87 498—506

Using precision calorimetric techniques for measuring extremity heat loss possible contra indications or advantages of alcoholic or hot drinks or smoking just prior to cold exposure were assessed Ten subjects were given 60 min exposures with one hand and one foot immersed in 15 C water following the drinking of 1 200 ml of 23 C water (control) 2 200 ml of 23 C water containing 22 ml of absolute alcohol 3 200 ml of 55 C bouillon 4 200 ml of 23 C water and smoking 2 cigarettes No significant effects were noted in the foot but significant differences were observed in hand heat loss to the calorimeter smoking depressed hand heat loss alcohol elevated it Smoking had a persistent influence but the effect of alcohol had disappeared after 30 min Bouillon had an effect similar to alcohol but of shorter duration also resulted in a significantly reduced heat loss after 30 min Alcohol produced a significantly higher amount of cold induced vasodilatation (CIVD) with greater total temperature elevation in 3 fingers than did smoking although smoking did not prevent CIVD With intake of bouillon CIVD appeared earlier than with alcohol and during the 1st 15 min total temperature elevation in the 3 fingers was markedly higher than in the control run but not as large as with alcohol All differences were relatively small and would seem to be of little practical importance

A considerable folklore exists as to the merits of alcoholic or hot drinks and the disadvantages of smoking prior to cold exposure Despite a reasonably substantial published literature there are few quantitative experimental studies there is some confusion between central and peripheral effects and there is considerable variation in the recommendations from a number of authoritative sources

Alcohol People have used alcohol to alleviate the effect of cold for centuries (Kohler 1942 Schwiegk 1950) although its efficacy has been disputed As a vasodilator alcohol increases the blood flow to the skin and thus produces a subjective sensation of warmth although actually increasing heat loss without increasing the resting heat production (Andersen *et al* 1963) Fortunately the increase in heat loss is usually only moderate The narcotic effect of alcohol on thermo-

regulatory centers in the brain may be more serious since shivering the normal compensatory reflex increase in heat production upon exposure to cold may be inhibited and a prolonged cold exposure could thus result in hypothermia Finally while the initially stimulating effect of alcohol may lead to increased physical activity and thus increased heat production both the accompanying impairment of judgment and the subsequent depression in activity produced by alcohol are serious drawbacks to its use The preceding statements are predominately theoretical (Andersen *et al* 1963 Kohler 1942 Schulze 1947 a)

Contrary to general belief intake of moderate amounts of alcohol neither significantly *increases total body heat loss in the cold nor decreases heat production* larger amounts of alcohol may reduce heat production by reducing shivering and this may eventually lead to hypothermia but only in resting or sleeping subjects (Ames *et al* 1948 Andersen *et al* 1963 Gupta 1960 Keatinge and Evans 1960 Schulze 1947 a Schwiegk 1950) Alcohol enhances the individual s tolerance of hypothermia once it has occurred (Blair 1964) although there may be a marked compensatory vasoconstriction when the effect of the alcohol wears off (Gupta 1960) Many authors ignore the preceding primarily subjective and/or central whole body effects of alcohol and emphasize the vasodilatation of peripheral areas most susceptible to frostbite and therefore recommend alcohol as a prophylactic in the cold (Kohler 1942, Schwiegk 1950) Accordingly during the second World War Russian soldiers were given 100 g of alcohol daily (Kohler 1942) and German soldiers received 125 ml of brandy (Schwiegk 1950) The vasodilator effect of alcohol administered as a hot drink is said to be intensified (Schulze 1947 a 1947 b)

Hot drink The maximum temperature at which a hot drink can be ingested appears to be 62° C in our experience Thus the maximum heat added to a 37° C body core by even this hottest drink can only be about 10 5 kilojoule per 100 ml of liquid ingested a relatively small amount of heat relative to the total body mass However swallowing a hot drink results in an immediate and pronounced peripheral vasodilatation (Blumberger and Glatzel 1961 Kramer and Schulze 1948 Schulze 1947 a Schwiegk 1950 Thoren and Folkow 1959) presumably as a reflex response to heat stimulation of sensors in the mouth and throat In one study (Kramer and Schulze 1948) a finger that had been cooled to 4° C in − 18° C air promptly rose to 30° C and stayed in that temperature range for 40 min as the result of immediate vasodilatation upon administration of 500 ml of a 60° C drink (Schwiegk 1950) It was suggested (Schwiegk 1950) that the risk of cold injury might be delayed by 1 h or more in this manner In another study (Schulze 1947 a) similar results were reported

Smoking Numerous measurements have shown that smoking one cigarette almost immediately decreases the circulation to the fingers and toes (Larson *et al* 1961 Miller and Bjornson 1962) the decrease in blood flow produces a fall in skin temperature which may amount to 1—2° C or even to as much as 7—8° C

of this effect smoking has generally been considered to increase the risk for frost bite However the situation is considerably more complicated Various factors modify the physiological response as discussed by Larson *et al* (1961), and make a straight forward statement impossible

The present study was undertaken to elucidate the effects that these three agents may have in reasonable amounts as experienced in daily life upon the heat loss from the hands and feet when exposed to a short period of moderate cooling

Material and Methods

10 healthy males (mean age 32 range 21—48 years mean body weight 72 kg) all smokers were each given 4 separate 60 min immersion exposures of one hand and one foot simultaneously in constant temperature water bath calorimeters at 15 C water temperature The immersion was preceded by a 45 min rest at a room temperature of 23 C in a recumbent position wearing shirt and trousers and a 15 min pre soak with the hand and foot in 34 C water The apparatus and methodology has been described previously (Newman and Breckenridge 1968) Any decrease in calorimeter heater power input corresponds quantitatively to an increase in heat input by the subject Skin temperatures were obtained from three digits (1st, 3rd and 5th) on the hand and correspondingly on the foot The digital temperature was measured by a small thermistor surface probe attached by waterproof tape on the dorsum of the terminal phalanx immediately proximal to the nail The probes were cycled in turn with 3 s intervals through a YSI thermistor thermometer (0 —40 C) and printed on a Leeds and Northrup Speedomax Model H

The total number of CIVD (cold induced vasodilatation) cycles for the three digits of the hand were counted in each subject Since the amount of heat loss per digit in each CIVD is a combination of the temperature elevation from a non CIVD base line and the duration in time the CIVD response was quantified by tracing the CIVD curves on the recorder chart with a planimeter This gave a reading in cm^2 for the area formed by the temperature rise and fall of each of the three fingers undergoing CIVD which was expressed as C×min All C×min shown by the three digits were added into one summation for each subject for each condition which gave the total temperature elevation in C×min The measure is relative because it is not known what heat loss is associated with a given C×min value since the hand surface area involved in CIVD at any one time cannot be estimated

During the 15 min pre soak the subject in randomized order did one of the following 1 drank 200 ml of room temperature (23 C) water 2 drank 200 ml of room temperature water containing 22 ml of absolute alcohol 3 drank 200 ml of hot (55 C) bouillon within 5 min or 4 drank 200 ml of room temperature water and smoked 2 cigarettes All subjects underwent an exposure under each of these conditions No food or smoking was allowed for 2 h before the start of an exposure nor was alcohol permitted after 2200 h on a night preceding an experiment Each subject was always tested at the same time of day to avoid possible diurnal cyclic effects

A subject by treatment analysis of variance was used to test for significant differences among these four conditions (Li 1964) If the F value was significant Tukey s method for calculating a critical difference for all pair comparisons was used This gives a value such that any two means differing by this amount or greater can be said to happen by chance less than 5 % of the time ($p < 0.05$)

Results

A comparison of the average rate of heat loss for the hand and foot in the water bath calorimeter for the 4 experimental conditions is shown in Fig 1 for 15 min intervals The mean values of the heat loss are presented in Table I for 15 min intervals and in Table II for the total immersion period

There were significant ($p < 0.05$) differences in hand heat loss in the expected directions, *i e* smoking depressed heat loss while alcohol elevated it and bouillon

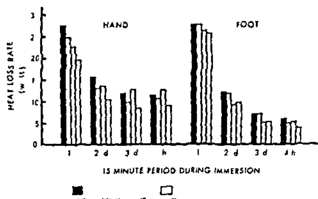


Fig 1 Mean rate of heat loss in 15 min intervals for the hand and the foot during 1 hour immersion in 15 C water

TABLE I Mean values (±1 S E) for the heat loss (watt) from the hand and from the foot for 15 min intervals during the water bath calorimeter exposure for the 4 experimental conditions in 10 men.

	Hand				Foot			
Minutes	0–15	15–30	30–45	45–60	0–15	15–30	30–45	45–60
Control	2? 4 ±1 7	13 ? ±2 2	12 5 ±2 2	12 3 ±1 6	25 9±1 4	9 3±0 8	5 2±0 3	5 2±0 2
Alcohol	27 4*±2 3	15 5 ±1 7	11 6 ±1 9	11 ? ±1 9	27 4±1 4	12 2±1 4	7 1±1 4	5 9±1 2
Bouillon	24 5 ±1 9	12 9 ±2 2	9 6 ±2 2	10 5 ±2 0	27 4±1 7	12 0±1 4	7 1±1 5	5 0±1 0
Smoking	19 3*±1 9	10 2*±2 2	8 1*±1 7	8 8* ±1 6	25 4±0 9	10 0±0 8	5 3±0 5	3 8±0 3

* Significantly different from the corresponding contr l period (p 0 05 critical d ff rence for hand values = 2 7 no s gnificant treatment eff cts for foot)

TABLE II Values for the total h at loss f om th hand and from the foot th total sum of CIVD for the 3 f ngers mea ured du ing e ch exposu e and the total temp ratu e elevat on exhib ted during CIVD cy l for the 3 f ng rs V lues for I–IV a e the mean (±1 S E) of 10 subject dur ng l hou e posu s Values for V and VI are th me ns (±1 S E) of the 1 t 15 min only

		Control	Alcohol	Bouillon	Smoking
I	Hand heat loss (watt)	15 1±1 9	16 5±1 5	14 4±1 9	11 6*±1 7
II	Foot heat loss (watt)	11 2 0 6	12 0±1 2	12 8±1 3	11 0 ±0 6
III	Total number f CIVD (3 f ng rs)	4 7±1 3	7 0±1 1	5 2±1 1	? 7 ±0 9
IV	Total temperatu e l at on (C m n) for 3 finger	9 3±10 6	61 ? 1? 0	37 4±9 ?	20 6 ±8 5
V	Tot l number of CIVD (3 fingers) du ng 1 t 15 min only	1 3±0 5	3 2 ±0 6	2 7±0 6	0 7 ±0 4
VI	Temp ratur levat on (C×m n) fo 3 fingers du ing 1 t 15 min only	4 8±2 6	23 4 ±5 6	15 7±3 1	3 9 ±? 0

S gnif cantly d ff ent fr m th correspond ng control period (p 0 05 r t cal diff rence fo I = 2 7 for III – 3 4 f IV = 31 6 for V = 1 6 for VI = 11 6 for II ther are no differences) Alcohol and smoking per ods diff r s g i fic ntly from each other fo I and III–VI

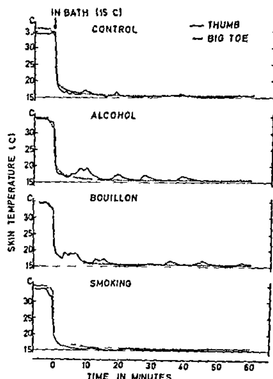


Fig 2 Typical temperature recordings of responses in skin temperature of the thumb (showing clear CIVD responses) and the big toe measured during each of the 4 trials in 1 subject

had a similar trend as alcohol The most consistent effect was caused by smoking which was evident in the total heat loss as well as in each of the 15 min periods The elevating effect of alcohol was only significant during the 1st 15 min during the 2 last periods the trend was even the opposite While bouillon seemed to increase the heat loss of the hand during the 1st 15 min period the following trend was a decrease in heat loss which was significantly lower ($p < 0.05$) than control during the 3rd period Whether this was due to a reflex vasoconstriction following an initial vasodilatation or purely coincidental is difficult to decide the trend of the changes in foot heat loss does not support the former contention No significant differences in foot heat loss between any of the experimental conditions were observed, neither in total heat loss nor during any of the 15 min periods

The mean values of the total number of CIVD cycles for the three fingers and the total temperature elevation (°C×min) exhibited during the CIVD cycles for the 3 fingers during the 60 min exposures are presented in Table II The increase in total temperature elevation after alcohol intake was the only significant ($p < 0.05$) change However it may be noted that the experimental periods with alcohol and smoking differ significantly ($p < 0.05$) from each other (although not consistently from the control period) with respect to total hand heat loss total number of CIVD cycles and total temperature elevation for the 3 fingers

Only 4 of the 10 subjects responded with CIVD during the 1st 15 min of the

control exposure while 9 of them showed CIVD during the same period after intake of bouillon as well as after alcohol Also the total temperature elevation for the 3 fingers during this time was markedly higher after intake of bouillon than in the control run although not as high as after alcohol ($p < 0.05$) as seen in Table II In half of the cases, CIVD appeared on an average 2 min earlier after hot bouillon than after alcohol in only one case was CIVD more rapid after alcohol 4 cases did not differ between the two The differences are apparent in Fig 2 showing typical temperature recordings with clear CIVD responses in skin temperature of the thumb and the big toe measured during each of the 4 trials in one subject

CIVD cycles did not occur in the toes during control exposures in any of the subjects but sometimes appeared after administration of bouillon (in 4 subjects) and alcohol (in 3 subjects) However it was felt that the toe re ponses were not marked enough to allow significant calculations

Discussion

It is evident from our results that smoking as well as alcohol and hot drink affects the heat loss from the hand While the increase in heat loss due to alcohol was significant only during the 1st 15 min the decrease in heat loss due to smoking was clearly evident during the entire 1 hour period This is somewhat surprising since only minor changes in vasoconstriction as an effect of tobacco were expected to occur at the temperature le el (15° C) used for the water bath The prolonged effect was unexpected because it is known that the maximum vasoconstrictive effect of tobacco resulting in a decrease in blood flow and a fall in skin temperature occurs only in completely dilated or non constricted blood vessels *i e* when body and skin are warm at an environmental temperature of about 25° C the effect of tobacco will be correspondingly less at lower temperature (Larson *et al* 1961) Also a decrease in room temperature from 28° C to 20° C results in a greater decrease in blood flow than smoking two cigarettes (Larson *et al* 1961 Shepherd 1963 Wood 1960) Greenfield (1963) has pointed out that the blood flow through the hand is at its lowest value at about 15° C and that CIVD is less conspicuous than at lower temperatures Further maximum vasoconstriction after smoking is said to seldom last more than a few minutes(Larson *et al* 1961 Weatherby 1942) though the effects of vasoconstriction may be noticeable up to 1 h

Although the increase in vasoconstriction due to smoking tended to decrease the number of CIVD cycles in the present investigation it did not inhibit the occurrence of CIVD Schulze (1947 b) also has found CIVD to occur with smoking Smoking at a normal rate of inhalation (~ 1 per min) does not seem to be accompanied by any significant decrease in hand blood flow (Larson *et al* 1961 Shepherd 1963) nor is vasoconstriction very marked (Rothman 1954 Schulze 1947 b Schwiegk 1950) but when cigarettes are smoked in excess of the normal rate as is usually the case under laboratory conditions the resulting decrease in blood flow pharmacologic and not physiologic in origin (Larson *et al* 1961 Shepl

This may be one explanation for the marked effect of smoking observed in the present study since the smoking rate was fairly rapid Smoking is also said to de crease the reactive hyperemia following cold exposure (Larson *et al* 1961, Weather by 1942 Wood 1960) indicating a protracted vasoconstrictive effect Smoking a cigarette induces presumably neurogenic vasoconstriction in the skin and a vasodila tation in the muscles presumed to be caused by epinephrine released from the adrenals (Larson *et al* 1961 Ruef *et al* 1955)

The significant increase in heat loss from the hand and total temperature eleva tion in the fingers after alcohol administration in our investigation agree with the previous findings of Schulze (1947 a) Keatinge and Evans (1960) and Gupta (1960) In contrast Ames *et al* 1948 Andersen *et al* 1963 and Schindl 1944 have failed to find vasodilatation as an effect of alcohol in the cold probably because of differences in total body thermal state between studies It is well known that the heat balance of a subject may markedly modify the vasomotor response to cooling (Greenfield 1963 Rapaport *et al* 1949 Schwiegk 1950) and a centrally timulated vasoconstriction produced by a negative heat balance will often override the stimulus for vasodilatation produced by alcohol (Keatinge and Evans 1960 Kramer and Schulze 1948 Schulze 1947 a Schwiegk 1950) On the other hand alcohol may temporarily inhibit vasoconstrictor tone (Kramer and Schulze 1948 Schulze 1947 a Thoren and Folkow 1959) and in at least one study (Kramer and Schulze 1948 Schwiegk 1950) has been shown to facilitate the occurrence of CIVD and probably thereby protect against cold injury during an hour or more of cold exposure (Schwiegk 1950) However a rather large amount of alcohol has to be used (Schulze 1947 a Schwiegk 1950)

Our observation that the effect of alcohol was most marked in the beginning of the cold exposure and then had an opposite trend was also made by Gupta (1960) The effect of the hot drink in our study was even more rapid and disappeared earlier than that of alcohol which is consistent with the view that it presumably is a reflex response Rothman (1954) has suggested that the response to alcohol is also a viscero-cutaneous reflex rather then a chemical pharmacological effect of the alcohol circulating in the blood stream

While alcohol and smoking clearly influenced the heat loss from the hand the heat loss from the foot was not significantly altered in the present study although CIVD in toes occurred in a few subjects after intake of alcohol and bouillon This agrees with the general observation that the peripheral circulation of the foot responds less to vasomotor stimuli than the hand (Rothman 1954 Schwiegk 1950) Accordingly there is considerably less vasodilatation in the foot than in the hand after alcohol administration (Horton *et al* 1936 Horwitz *et al* 1949 Rothman 1954 Schulze 1947 a although alcohol seems to increase the toe temperature in the cold (Little 1970) The effect of smoking is also less in the foot (Larson *et al* 1961 Simon *et al* 1954) The smaller degree of heat loss from the toes and foot during immersion in cold water is due to a smaller blood flow (Greenfield 1963 Kunkel and Stead 1938 Shepherd 1963) There is also less CIVD response in the foot as a

result of greater vasoconstrictor tone in the lower as compared to the upper extremity (Shepherd 1963)

In conclusion while this study has demonstrated negligible changes for the foot both alcoholic and hot drinks have been shown to have initially a positive effect while smoking was found to have a negative effect on the heat input to the hand However the observed average differences in heat loss were so small that there would seem to be little practical effect from the amounts administered at least not for water immersion exposure at 15° C

The work of Ove Wilson was supported by the Swedish Medical Research Council Project B 0-40P 2940-01) and the Swedish Delegation for Applied Medical Defence Research Project U16/69)

References

AMES A R S GRIFFITH D A GOLDTHWAIT M B MACHT and H S BELDING An Evaluation of Methods of Rewarming Men Environ Protect Ser Rept No 134 Quartermaster Climatic Research Laboratory Lawrence Mass 1948

ANDERSEN K L B HELLSTROM and F V LORENTZEN Combined effect of cold and alcohol on heat balance in man J appl Physiol 1963 18 975—982

BLAIR E Clinical Hypothermia McGraw Hill New York 1964 pp 77—181

BLUMBERGER W and H GLATZEL Kreislaufregulation and Ernahrung I Die Beeinflussung der Hautdurchblutung durch heisse Getranke Z Kreisl Forsch 1961 50 357—372

FRANKE H and J SCHRODER Zum Problem der Wirkung des Kauchens auf das periphere Gefasssystem bei Gesunden und Kranken Dtsch Arch klin Med 1955 202 320—346

GREENFIELD A D M The circulation through the skin In Handbook of Physiology Circulation Sect 2 Vol II chapt 39 pp 1325—1351 Williams & Wilkins Baltimore 1963

GUPTA K K Effect of alcohol in cold climate J Indian med Ass 1960 35 211—218

HORTON B T M ROTH and A W ADSON Observations on some differences in the vasomotor reactions of the hands and feet Proc Mayo Clin 1936 11 433—437

HORWITZ O H MONTGOMERY E D LONGAKER and A SAYEN Effects of vasodilator drugs and other procedures on digital cutaneous blood flow cardiac output blood pressure pulse rate body temperature and metabolic rate Amer J med Sci 1949 218 669—682

KEATINGE W R and M EVANS Effect of food alcohol and hyoscine on body temperature and reflex responses of men immersed in cold water Lancet 1960 II 176—178

KOHLER O Erfahrungen uber die Behandlung von Frostschaden Zbl Chir 1942 69 1782—1794

KRAMER K and W SCHULZE Die Kaltedilatation der Hautgefasse Pflugers Arch ges Physiol 1948 250 141—170

KUNKEL P and E A STEAD JR Blood flow and vasomotor reactions in the foot in health in arteriosclerosis and in thromboangiitis obliterans J clin Invest 1938 17 715—723

LARSON P S H B HAAG and H SILVETTE Tobacco Experimental and Clinical Studies Williams & Wilkins Baltimore 1961 pp 216—232

LI C C Introduction to Experimental Statistics McGraw Hill New York 1964 p 425

LITTLE M A Effect of alcohol and coca on foot temperature response of highland Peruvians during a localized cold exposure Amer J Phys Anthrop 1970 32 233—242

MILLER D and D R BJORNSON An investigation of cold injured soldiers in Alaska Milit Med 1962 127 247—252

NEWMAN R W and J R BRECKENRIDGE A constant temperature water bath calorimeter for measuring extremity heat loss J appl Physiol 1968 25 447—449

RAPAPORT S I E S FETCHER H C SHAUB and J F HALL Control of blood flow to the extremities at low ambient temperature J appl Physiol 1949 2 61—71

ROTHMAN S Physiology and Biochemistry of the Skin University of Chicago Press Chicago—London 1954 pp 104 233—264

RUEF J K D BOCK and H HESSE Über die Wirkung des Rauchens auf die Muskeldurchblutung Z Kreisl Forsch 1955 44 272—28

SCHINDL K Hauttemperaturen bei Kalteeinwirkungen Z ges exp Med 1944 113 613—6

SCHULZE W Untersuchungen uber den Einfluss des Alkohol auf die periphere Durchblutung bei lokaler Kalteeinwirkung Klin Wschr 1947 a 25 646—654

SCHULZE W Über den Einfluss des Rauchens auf die periphere Durchblutung bei lokaler Kalteeinworkung *Klin Wschr* 1947 b *25* 738—742
SCHWIEGK H Pathogenesis and treatment of local cold injury In *German Aviation Medicine World War II* vol 2 pp 843—857 Department of the Air Force Washington D C 1950
SHEPHERD J T *Physiology of the Circulation in Human Limbs in Health and Disease* Saunders Philadelphia—London 1963 pp 101—126 381—389
SIMON D L A IGLAUER and J BRAUNSTEIN The immediate effect of cigarettes on the circulation of healthy and habitual male smokers A study in ballistocardiography *Amer Heart J* 1954 *48* 185—188
THOREN O and B FOLKOW Forsok med karldilaterande salva sasom skydd mot forfrysning *Tidskr milit Halsovard* 1959 *84* 85—97
WEATHERBY J H Skin temperature change caused by smoking and other sympathomimetic stimuli *Amer Heart J* 1942 *24* 17—30
WOOD J E Effect of smoking on the peripheral circulation in relation to environmental temperature *Ann N Y Acad Sci* 1960 *90* 114—118

Acta physiol scand 1973 87 507—513

From the Department of Clinical Physiology Karolinska Hospital Stockholm Sweden

A Respiratory Reflex Elicited by Hemorrhage and Mediated by Vagal Afferents

By

TORGNY SJOSTRAND

Received 11 July 1972

Abstract

SJOSTRAND T *A respiratory reflex elicited by hemorrhage and mediated by vagal afferents* Acta physiol scand 1973 *87* 507—513

Ventilation heart rate and arterial pressure were recorded in rats on blood losses from 1 to 6 ml (6—36 % of blood volume) About 15 s after the onset of the blood drawing and the coincident decrease of arterial pressure the tidal volume increased on a loss of 1—3 ml This was regularly observed in 10 rats On larger hemorrhages the tidal volume decreased The respiratory rate decreased in some animals already at a blood loss of 1 ml in others at losses above 2 ml The increase of the tidal volume was totally inhibited in 5 rats by exclusion of afferents in the recurrent nerves by cold block or section In three other rats the increase of the tidal volume was inhibited on cutting also of the right or both vagal nerves The decrease of tidal volume and of respiratory frequency on large blood losses was not inhibited by vagotomy It is presumed that the increase of the tidal volume on a small to moderate blood loss is provoked by a vagal reflex elicited by receptors on the low pressure side and that the decreased ventilation on larger hemorrhages is elicited from the respiratory centres by the change of the cerebral blood flow

Respiratory reflexes elicited from the vascular system by hemodynamic changes have been suggested However in a critical review of the literature Widdicombe (1964) established that evidence for an influence of the arterial baroreceptor reflex on the ventilation has been adduced but other presumed reflexes from the cardiovascular system are not sufficiently authenticated It is well known that respiration is changed on hemorrhage but this may have various causes such as metabolic acidosis liberation of adrenaline stimulation of chemoreceptors or a direct action on the respiratory centres (see Wiggers 1950) Thus a proof of a cardiovascular origin of the changes must be based on an immediate connection between the hemodynamic change and the respiratory response

In a study of the circulatory effect of variations of the central blood volume in Sprague Dawley rats it was observed that the ventilation changes on drawing of blood An analysis of this phenomenon indicates the existence of a reflex elicited by a decrease of the central blood volume and affecting the depth and frequency of the ventilation

Material and methods

Sprague Dawley rats (275—370 g weight) anesthetized with sodium pentobarbitone i.p 5—8 mg/100 g b wt were used

In pilot experiments the ventilation was recorded by applying a strain gauge string around the thorax To exclude the effect of changes of the thoracic mid position a body box in 2 pieces was later constructed of plastic with a hole for the tracheal cannula 3 tubes were passed through the wall for connection to arterial catheters and for recording pressure changes in the box A connecting contact was introduced in the wall for the ECG recording and temperature control 2 tubes were inserted through the cover for the cooling liquid The body box was sealed with sealing wax The box was not air tight which would have produced volume changes on drawing of blood and cooling of the nerves The volume references are consequently approximative

Blood pressure and the pressure in the box were recorded with Elema differential transformer transducers (E M T 490 A) Catheters were inserted in both femoral arteries for recording the pressure and for drawing of blood Heart rate was determined from the ECG fed through a heart rate detector with a time constant of 2 5 s (Sonus) The recording instrument was a direct ink writer (Watanabe)

A cold block of the recurrent nerves was obtained by applying a metal tube around the trachea A perfusion pump was used to perfuse the tube with acetone cooled with iced carbon dioxide A thermistor was placed on the trachea close to the tube The temperature was observed on an ammeter On moving the thermistor 1—2 mm from the contact with the tube the recorded temperature increased 10 —15 C Cooling of the vagal nerves was thus excluded It was also found that this would demand a much lower temperature at the tube and a longer freezing time No significant changes in respiration were observed during cooling of the nerves

Procedure

In the experiments with the body box respiration blood pressure and heart rate were recorded during 5 min after closure of the box Blood was drawn rapidly and usually reinfused when an approximately constant pressure level was reached This was repeated several times sometimes with drawing of varying blood volumes Thereafter the cooling liquid was perfused and the temperature of the trachea was observed When about 0 C was reached the perfusion was adjusted to maintain the temperature Blood was then drawn repeatedly After stopping the perfusion and when the temperature of the trachea had reached the initial value blood was drawn off again This procedure was usually repeated several times in each animal

Results

Respiratory changes on drawing of varying amounts of blood

Fig 1 demonstrates the effect on heart rate arterial pressure and ventilation on drawing of 1 and of 3 ml blood from the femoral artery in the same rat During the fall of the arterial pressure the tidal volume increases and the respiratory rate decreases as the heart rate The change of ventilation starts about 15 s after the onset The increase of tidal volume on the smaller blood loss is about half of that on the larger In both experiments the respiratory changes begin at an arterial pressure of about 80 mm Hg

Fig 2 demonstrates the percentual changes of tidal volume and respiratory frequency on successive drawing of blood in three rats The increase of the tidal volume reaches its maximum after a loss of 2 or 3 ml On a further blood loss it declines to the initial level or lower The respiratory rate is unchanged in two rats and decreases in the third on a loss of 1 and 2 ml and then declines markedly in all rats Ususally the more pronounced decline succeeds a brief period of increased respiratory rate simultaneously with an increase or a decrease of the tidal volume Briefly after reinfusion the tidal volume and respiratory frequency are somewhat lower than before in the experiments with large blood loss

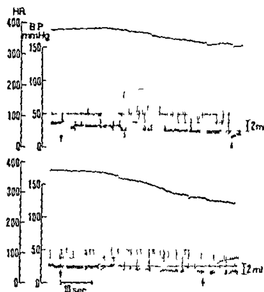


Fig 1 The effect on heart rate arterial pressure and ventilation of drawing of 1 (upper) and 3 (lower) ml blood from the femoral artery in a Sprague Dawley rat. During the fall of the arterial pressure the tidal volume increases simultaneously with a decrease of the respiratory rate

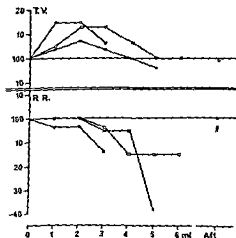


Fig 2 Percentual changes of tidal volume and respiratory frequency on successive drawing of blood and reinfusion

The increase of the tidal volume on a blood loss up to 3 ml (about 18 % of the blood volume) has been a constant phenomenon in experiments on in all 10 rats The effect lasts for at least 12 minutes if reinfusion is not done but tends to decrease slowly

It appears from Fig 2 that the increase of the tidal volume on small to moderate hemorrhage is at its maximum at a blood loss of 2—3 ml and the coincident decrease of the respiratory rate is established at 3 ml To analyse both effects drawing of around 3 ml blood was chosen The depressing effect of larger hemorrhages was studied in two rats by drawing off 5 and 6 ml respectively

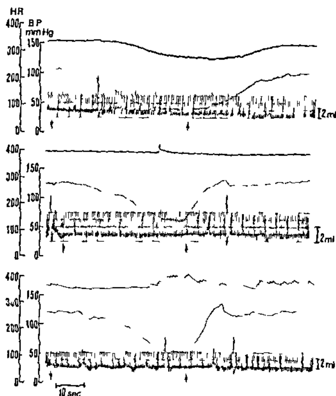


Fig 3 Heart rate arterial pressure and ventilation on a Sprague Dawley rat before and during drawing of 3 ml blood (at the arrow) and on reinfusion Before (upper) during (middle) and after (lower) cold block of the recurrent nerves

The effect of exclusion of vagal afferents on the respiratory changes on hemorrhage

In pilot experiments on 3 rats with strain gauge strings around the thorax it was found that the increase of the tidal volume on a moderate blood loss disappeared in 2 animals on cutting of the recurrent nerve and/or the superior laryngeal nerve bilaterally

To guarantee that this effect is not due to changes of the mid position of the thorax or to the operative procedure in itself the experiments were repeated using a body box and reversal blocking of the laryngeal pathway by cooling of the recurrent nerves

Fig 3 demonstrates the effect on heart rate, arterial pressure and ventilation on drawing off and reinfusion of 3 ml blood in a rat before during a cold block of the recurrent nerves and after rewarming the nerves The effect of the blood loss is the same as demonstrated in Fig 1 before the recurrent nerves are cooled On reinfusion the changes of arterial pressure heart rate and ventilation return fairly in parallel On cooling the nerves the arterial pressure drops somewhat less and the heart rate tidal volume and respiratory rate remain unchanged After rewarming the arterial pressure falls more than on cooling but somewhat less than before The tidal volume increases and the ventilatory rate decreases as at the start of the experiment but somewhat less pronouncedly The heart rate increases on hemorrhage but the record is artificially disturbed

TABLE I Percentual changes of tidal volume respiratory frequency and heart rate on hemorrhage before during and after cold block of the recurrent nerves in 3 rats (1–3) In rats 4–6 only a partial effect was obtained by cold block as also by laryngeal denervation but right (4 and 6) and also left vagotomy (5) brought about a full effect With a few exceptions the values are means of two successive experiments

Animal no	Condition	Blood loss ml	Tidal vol change %	Respiratory rate change	Heart rate change
1	Before	3	+19	− 9	− 7
	Cold block	3	± 0	− 2	+18
	After	3	+16	− 6	− 6
2	Before	3 5	+25	−11	−12
	Cold block	3 5	+ 1	− 7	+ 4
	After	3 5	+15	− 4	− 7
3	Before	3	+15	−16	−30
	Cold block	3	+ 0	− 8	− 2
	After	3	+15	−12	
4	Before	3 5	+25	+ 6	
	N recurr cut bilat	3 5	+16	+ 6	
	R vagus cut	3 5	− 5	−18	
	Vagotomy bilat	3 5	− 2	−15	
5	Before	2 4	+17	− 2	− 8
	After lar denerv	2 4	+ 7	± 0	− 4
	R vagus cut	2 4	+ 8	− 6	
	Vagotomy bilat	2 4	+ 1	± 0	
	Vagotomy bilat	6	± 0	−40	
6	Before	3	+24	− 7	− 1
	Cold block	3	+17	− 7 7	− 5
	R vagus cut	3	± 0	± 0	− 5
	Vagotomy bilat	3	−26	± 0	
	Vagotomy bilat	6	−23	−14	

In Table I data from experiments on six rats are compared In 3 rats the cooling of the recurrent nerve completely blocks the increase of the tidal volume of the moderate bloss loss The decrease of the respiratory rate is less after cooling the nerves but not completely blocked In the other 3 animal the increase of the tidal volume is slightly less after cooling The experiment was repeated after cutting of the laryngeal pathway which had the same effect as the cold block The heart rate varies between the animals as the effect on the tidal volume Cutting the right vagus inhibits the increase of the tidal volume in 2 rats In the third rat both vagal nerves had to be cut to exclude the response The changes of the respiratory rate are varying

It emerges from Table I also that the decrease of the tidal volume and respiratory frequency on drawing off more than 3 ml blood is not inhibited by vagotomy

Discussion

The parallel increase of tidal volume and decrease of arterial pressure and heart rate on a small to moderate blood loss indicate a reflex action The respiratory rate may be markedly decreased simultaneously with the increase of the tidal vol

but may also be unchanged on a small blood loss The decrease is obviously a reflex phenomenon and not an effect of a secondary adaptation of the ventilation On a large hemorrhage the tidal volume as well as the respiratory rate are decreased On gradual blood drawing an intermediate brief period with increased respiratory rate and increased or decreased tidal volume may appear The effect of small or moderate hemorrhages on the tidal volume is blocked by partial or complete exclusion of vagal afferents but not the effect of larger blood losses This is consistent with the supposition of at least two separate mechanisms provoked by hemorrhage one appearing on small or moderate blood losses mediated by vagal afferents and the other provoked by larger blood losses and not dependent on vagal afferents or efferents It seems most plausible to ascribe the latter effect to the influence of changes of the cerebral blood flow on the respiratory centres

The suggested vagal respiratory reflex corresponds to the earlier described reflex change of the heart rate on hemorrhage in some rats wholly mediated via afferents in the laryngeal communicans (Castenfors and Sjostrand 1972) In other animals the reflex is mediated also by afferents in the vagal trunk and/or cardiac nerves

The effect of exclusion of the laryngeal pathway on ventilation and heart rate has varied in the same way between different Sprague Dawley rats It is therefore presumed that these effects are elicited by the same receptors which have been supposed to be localised to the low pressure side of the circulation (Castenfors *et al* 1972) The possibility of an effect of the chemoreceptors in the aortic body may also be considered Landgren and Neil (1951) observed an increased activity in vagal multifibre preparations (as in the sinus nerve) on hemorrhage to an arterial pressure of 40 mm Hg in the artificially ventilated cat They believed that the discharges erived from chemoreceptors in the aortic body because they appeared in small fibres They draw the conclusion that these receptors (as also the chemoreceptors in the carotid sinus) are stimulated by metabolic or circulatory changes in the aortic body (carotid body respectively) They did not analyse the blood for pO_2 pCO or pH Their suggestion concerning the carotid chemoreceptors has not been confirmed by Biscoe *et al* (1970) who kept the blood gases and pH constant The criticism of these authors is valid also as regards the stimulation of the aortic body Lee *et al* (1964) studied the effect of changes of the aortic pressure on the activity in a few fibre preparations of nerves from the aortic body and made some observations on hemorrhage They found *inter alia* that the activity at ordinary pressure dropped to a fifth on a change of the inspiratory gas mixture to the respiratory pump from air to 30 % O in N and that the activity increased five times on a reduction to 14 % On hemorrhage the activity increased but the relationship between the 3 conditions was maintained They did not present any absolute values of pO pCO or pH—only the variability of pO on occlusion of the aorta or common carotids The most plausible explanation of their observations on hemorrhage seems to be that the ventilation/perfusion ratio varied in different parts of the lungs and that the most ventilated parts exposed to the highest pressure were least perfused On a decrease of the pulmonary arterial pressure as on hemorrhage these parts would be still less perfused

Under these conditions the activity would vary with the O pressure of the inspiratory gas at ordinary vascular pressures It is thus not proved that the aortic chemoreceptors are stimulated directly on hemorrhage

Stimulation of the chemoreceptors as the cause of the ventilatory changes described in the present paper is opposed by the following findings 1 the increase of the tidal volume appears simultaneously with decreased or unchanged respiratory rate 2 the effect appears directly on a fall of arterial pressure and already at rather small changes 3 the effect appears coincident with a decrease of the heart rate (a stimulation of the chemoreceptors would provoke an increase) 4 on recording of the activity in the laryngeal pathway in 60 rats no activity has been observed which could be referred to asphyxia but in about half of the experiments the activity increased on hemorrhage

The functional significance of an increase of the tidal volume on hemorrhage is apparent The decrease of the central blood volume would be counteracted by the increased effect of the respiratory pump This effect may therefore be added to the effects earlier ascribed to receptors on the low pressure side such as a decrease of the heart frequency and an increase of the tone of the capacity vessels (Castenfors *et al* 1972) All these effects would contribute to maintaining the central blood volume at an optimal level

A reflex activation of the respiratory pump on a decrease of the central blood volume may appear also under other conditions with decreased central blood volume In the dumping syndrome and in early stages of circulatory chock the ventilation is increased (Castenfors *et al* 1962 Wiggers 1950) Also the hyperventilation on exhaustive exercise may be the result of a reflex activating the respiratory pump in order to maintain the central blood volume

References

BISCOE T J G W BRADLEY and M J PURVES The relation between carotid body chemoreceptor discharge carotid sinus pressure and carotid body enous flow *J Physiol* (Lond) 1970 *208* 99—120

CASTENFORS J and T SJOSTRAND Circulatory cont ol v a vagal afferents I Adjustment of heart rate to variat ons of blood olume in the rat *Acta physiol scand* 1972 *84* 347—354

CASTENFORS H L G EKELUND and A HOLMGREN Effect of ingestion of hyperosmotic glucose solution on the system c and splanchn c c rculation in partially gastrectomized patients w th histories of dumping syndrome *Scand J cl Lab Invest* 1962 *14* 329—342

CASTENFORS J E KNUTSSON and T SJOSTRAND Circulatory control v a agal afferents II Effect of hemorrh ge on afferent discharges via the laryngeal communicans in the rat *Acta physiol scand* 1972 *84* 355—365

CASTENFORS J E KNUTSSON and T SJOSTRAND Circulatory control via vagal affe ents III Control of arterial blood p essure by afferents via the laryngeal commun cans ith special regard to the rest tut on after hemo hage *A ta phy l sca d* 1973 *87* 176—187

LANDGREN S and E NEIL Chemo eceptor mpulse activity following hemorrhage *Acta phys ol sca d* 1951 *23* 158—167

LEE K D R A Mayou AND R W TORRANCE The effect of blood pressure upon chemoreceptor d scharge to h poxia and the modif cation of this effect by the sympathetic adrenal system *Qua t J exp Physiol* 1964 *49* 171—183

WIDDICOMBE J G Respiratory reflexes In *Ha dbook of phys logy* Sect 3 Respiration vol 1 pp 585—630 Washington DC Ame Physiol Soc 1964

WIGGERS C J *Phy ology of shock* Harvard Un ersity press Cambridge Mass USA 19

Acta physiol scand 1973 87 514—525

From the Institute of Physiology, University of Bergen and the Institute for Experimental Medical Research University of Oslo Ulleval Hospital Norway

Effect of Hemorrhagic Hypotension on the Distribution of Renal Cortical Blood Flow in Anesthetized Dogs

By

K Aukland A Kirkebo E Loyning and I Tyssebotn

Received 18 July 1972

Abstract

Aukland K, A Kirkebo E Loyning and I Tyssebotn *Effect of hemorrhagic hypotension on the distribution of renal cortical blood flow in anesthetized dogs* Acta physiol scand 1973 87 514—525

The distribution of renal cortical blood flow was studied in seven Nembutal anesthetized dogs in control periods during hemorrhagic hypotension and after retransfusion Local cortical blood flow was measured as hydrogen clearance recorded simultaneously from 3 to 6 platinum electrodes inserted into inner and outer half of the cortex Total renal blood flow (RBF) was measured by electromagnetic flowmeter In control periods flow averaged 3 47 ± 0.83 ml/min × g in outer cortex and 3 61 ± 0 86 ml/min × g in inner cortex Bleeding to mean arterial pressure of 50 mm Hg in the course of 5—36 min in 5 expts caused a proportional reduction of RBF and average flow in outer and inner cortex to 40—60 % of control with further reduction to 10—30 % with maintenance of hypotension for 2 h Bleeding to 30 mm Hg in 2 expts gave principally the same response All electrodes showed a similar response and the percentual deviation between electrodes did not increase during hypotension Thus no evidence was obtained for patchy or segmental ischemia or for regional redistribution of cortical blood flow Retransfusion gave only incomplete restoration of flow

---

Hemorrhagic hypotension is known to cause a marked reduction of the total amount of blood flowing through the kidney but there is no agreement as to whether the ischemia is general or mainly located to certain zones of the organ The idea advanced by Trueta *et al* in 1947 on the basis of India ink injections that the cortical circulation is especially susceptible to ischemia has recently received support from experiments based on more elaborate methods Carriere *et al* (1966) and Truniger Rosen and Oken (1966) using external recording of the clearance of Xe^{133} injected into the renal artery of dogs and Kr^{85} autoradiography concluded that hemorrhagic hypotension leads to marked reduction of blood flow in the outer part of the cortex (sometimes patchy) with unchanged or even increased flow through the juxtamedullary cortex and outer medulla —However other investigators have reported ischemia also in the medulla Measuring medullary transit time for albumin Kramer and Deetjen (Kramer 1962) found that medullary blood flow fell in pro

portion to total RBF during hemorrhagic hypotension Aukland and Wolgast (1968) confirmed this finding by measuring local medullary clearance of H and Kr[5] and local red cell transit time —They concluded that on the average cortical and medul lary blood flow was reduced proportionally but they had too few local measurements in the cortex to draw any definite conclusions about possible redistribution of blood flow within the cortex

A proportionate fall in deep and superficial cortical flow during bleeding was also observed by Logan *et al* (1971) using microsphere technique which reflects distribution of glomerular flow However such a proportionate fall in flow was observed after cannulation and ligation of one carotid artery a procedure which had also been used by Aukland and Wolgast (1968) When carotid artery ligation was avoided control flow was higher in the outer cortex than in the inner cortex and the former fell relatively more during bleeding

Inert gas clearance and microsphere localization obviously reflect flow in dif ferent cortical vessels The present experiments were undertaken in order to study the distribution of renal blood flow by measuring local H clearance during hemor rhagic hypotension in anesthetized dogs

Methods

7 expts were performed on 5 mongrel dogs weighing 10—20 kg The animals had free access to water whereas food was withdrawn 15 h before surgery The experiments were performed in Nembutal anesthesia (25 mg/kg) and the dogs were anticoagulated with 1 ml of heparin (5000 I U /ml) An endotracheal tube was inserted to secure free airways

Polyethylene tubes were introduced into a brachial vein for infusions and in a brachial artery for pressure recording with a Hewlett Packard transducer connected to a Hewlett Packard recorder A widebore cannula was inserted into a femoral artery and connected to a reservoir

The kidney and the renal artery were gently exposed retroperitoneally through a flank in cision taking care to avoid damage to the renal nerves A polyvinyl catheter was sewn into the renal artery as described by Herd and Barger (1964) A flowprobe was placed between the renal artery catheter and aorta, allowing continuous recording of total renal blood flow (RBF) with a Nycotron electromagnetic flowmeter Direct calibrations of the flowprobes were made in separate experiments on femoral or carotid arteries The ureter was dissected free and can nulated with a polyethylene tube

6 platinum electrodes were inserted into the renal cortex 3 in the outer half of the cortex 1—3 mm deep and 3 in the inner half 5—8 mm deep The tapered exposed electrode tip was about 1 mm long and 0 3 mm thick The L shaped shaft of the electrode was sutured to the capsule of the kidney with 2 sutures as previously described (Aukland 1968) The platinum electrodes were polarized with a potential of +0 2 V relative to a Ag/AgCl electrode inserted s c on the back of the dog The electrodes were then covered with perirenal fat and the flank incision was temporarily closed

Electrode current was measured by a 6 channel polarography amplifier lightly modified from that described earlier (Aukland 1968) and recorded on a Rikadenki potentiometer recorder (Rikadenki Kogyo Co Model B 64)

Hydrogen gas was administered in the renal artery to the experimental kidney by injecting 10—15 ml 0 9 % saline saturated with hydrogen gas The injection was given by hand and the rate adjusted according to electrode current so that cortical H_2-gas concentration was kept stable at a suitable level (about 5 % saturation) in the course of 1—2 min The washout curves following sudden stop of the injection were recorded until return of electrode current to control level

Para amino hippuric acid (PAH) was given iv at the end of the operation Fifty ml 0 32 % PAH in isotonic saline was used as a priming dose followed by a constant infusion of a 0 15 % PAH solution at a rate of 2 ml/min until the bleeding was started The total amount

did not exceed 200 ml. Urine sampling in 2 consecutive 10 min periods was started 40—60 min after completion of the surgery, arterial blood samples being collected in the middle of each period. The concentration of PAH in plasma and urine was determined by the method of Smith *et al.* (1945). Renal plasma flow was estimated from PAH clearance by assuming an extraction of 80 %. Comparison to RBF measured with electromagnetic flowmeter showed agreement within ±10 %.

Control measurements of local cortical blood flow were made before and after PAH clearance measurements, the last one or two controls before bleeding being recorded after i.v. injection of 1 ml heparin (5000 I.U./ml). The last H_2 washout rate from each electrode before bleeding was used as reference for later measurements.

In 5 expts. the dogs were bled to a mean arterial pressure ($\overline{AP}$) of 50 mm Hg in the course of 5—36 min and a measurement of cortical flow was performed at $\overline{AP}$ of 70—85 mm Hg. In 2 expts. $\overline{AP}$ was first stabilized at about 70 mm Hg, allowing 4 to 6 measurements at this pressure level. $\overline{AP}$ was then lowered rapidly by further bleeding, reaching 50 mm Hg 57 and 135 min after start of bleeding. In 5 animals the pressure was then maintained at this level for at least 2 h and in the last 2 for 70 min by slight adjustments of the height of the blood reservoir. Cortical flow measurements were made every 15—20 min. Retransfusion of the shed blood was completed in 15 to 20 min by elevating the blood reservoir. Blood flow measurements were made during and after retransfusion.

At the end of each experiment the kidney was removed and allowed to drain freely through ureter and renal vessels. The position of each electrode tip was located by careful dissection. Four electrodes were discarded because of close contact with macroscopic arteries or calyces or because of hematoma around the electrode tip. The kidney was finally weighed.

Flow calculation. When plotted on semilogarithmic paper most desaturation curves were practically linear down to 10 % of initial concentration or lower. 4 out of 36 electrodes in anatomical satisfactory position showed consistently marked deviation from linearity during washout of the first 90 % of the gas. Since no satisfactory method is available for calculating blood flow from multiexponential curves (Aukland *et al.* 1967), all curves recorded from these 4 electrode sites were discarded. From the remaining 32 electrodes, 19 in outer and 13 in inner cortex, local flow in ml/min×g was calculated as $0.693/T_{1/2}$, where $T_{1/2}$ is the half time in minutes.

Results

Control measurements. In prebleeding control periods mean arterial pressure was stable at levels between 100 and 120 mm Hg. Total renal blood flow (RBF) varied considerably between animals, from 90 to 380 ml/min or expressed per g weight of the excised and drained kidney from 4.0 to 6.7 ml/min×g with an average of 5.3 ml/min×g. Considerable variation was also observed in local cortical blood flow. As described under methods 4 electrodes in anatomical satisfactory position were excluded because of multiexponential desaturation curves. The remaining 32 electrodes gave practically monoexponential curves with a high degree of reproducibility, the coefficient of variation (SD in per cent of mean flow) between consecutive control measurements being only 3 %. However, considerable variation was observed between simultaneous recordings from different cortical electrodes in the same kidney. Comparison of electrodes irrespective of localization within the cortex showed SD of 0.62 ml/min×g corresponding to a coefficient of variation of 18 %, whereas comparison of electrodes within outer an inner cortex gave coefficients of variation of 14 and 24 % respectively. For comparison of different animals the mean of simultaneous flow recordings with one to three electrodes in each cortical layer was calculated ($\overline{OCF}$ and ICF). Control measurements in the 7 dogs showed following average: Outer cortex 3.47 (SD 0.83), inner cortex 3.61 (SD 0.86) ml/min×g. The

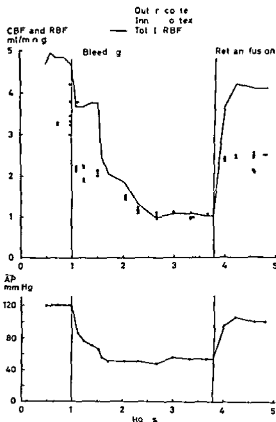


Fig 1 Effect of bleeding and ret ans fusion in one an mal on total renal blood flow (RBF) and local cort cal flow (CBF) at 6 diffe ent electrode sites $\overline{AP}$ Mean arterial pressu e

great scatter around mean obviously reflected real differences between animals as evident from Fig 2 where the control measurements in both cortical layers (en circled symbols) show fairly good correlation to total renal blood flow but an average cortical flow/RBF ratio of only 0 67 Furthermore Fig 3 shows reasonably good agreement between control measurements of outer and inner cortical flow in different animals In the last prebleeding control measurement the $\overline{OCF}/\overline{ICF}$ ranged from 0 74 to 1 29 with an average of 0 97 which is not statistically significant from one (Fig 5)

Hemorrhagic hypotension All dogs were ultimately bled to $\overline{AP}$ 50 mm Hg but the time used for reaching this pressure level was purposely varied in different animals However renal hemodynamical changes were basically the same in all experiments and they will therefore be considered together Detailed data from an experiment in which AP was lowered rather slowly are shown in Fig 1 Cortical flow fell gradually at all electrodes in outer and inner cortex to about 1/4 of co after two hours at an AP of 50 mm Hg Apart from the difference in absolute

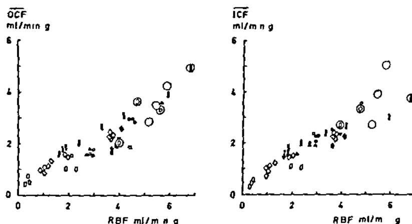


Fig 2 Mean outer ($\overline{OCF}$) and inner ($\overline{ICF}$) cortical blood flow related to total RBF in control periods (*encircled symbols*) *during hemorrhagic hypotension* (*open symbols*) and after retransfusion (filled symbols) Individual experiments represented by different symbols

the local flow pattern agreed well with RBF measured with electromagnetic flow meter Retransfusion gave only incomplete restoration of blood flow

In order to compare different electrode sites and different experiments local blood flow was calculated in per cent of the last control measurement before bleeding as shown in Table I In this experiment $\overline{AP}$ was first lowered to about 70 mm Hg and then 1 1/2 h later to 50 mm Hg Also this bleeding procedure produced a fairly

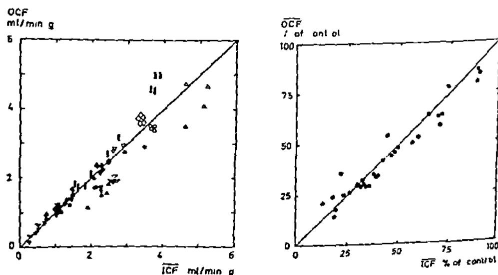


Fig 3 Mean blood flow in outer cortex related to flow in inner cortex in different dogs Filled symbols Hypotensive period Open symbols Control period Different symbol-shapes Different dogs

Fig 4 Relationship between outer and inner cortical flow ($\overline{OCF}$ and $\overline{ICF}$) during hemorrhagic hypotension, both in per cent of control measurements.

TABLE I Cortical flow distribution during hemorrhage and retransfusion

Time	$\overline{AP}$	RBF		Outer cortical blood flow					
				E_1	E_2	E_3		E	
min	mm Hg	ml/ min g		ml/ min g		ml/ min g	%	ml/ min g	
−4	110	5 47	100	3 00	100	3 46	100	3 95	100
0	Start bleed ng								
16	80	4 60	84	2 37	79	3 07	89	2 60	66
27	75	4 40	81	2 37	9	3 19	92	3 32	84
31	75	4 73	87	2 37	79	3 19	92	3 19	81
56	68	2 98	55	1 63	54	2 08	60	2 18	55
68	68	2 98	55	1 66	55	1 97	57	1 54	39
96	68	2 57	47	1 63	54	1 89	55	1 66	42
115	67	2 84	52	1 84	61	2 08	60	0 92	23
135	57			1 02	34	1 15	33	0 90	23
168	52			0 83	28	1 38	40	0 83	21
180	Start retransfusion								
192	Retransf is on complete								
195	110	4 26	78	2 08	69	2 86	83	2 96	75

proportional reduction of local blood flow at all electrode sites and of total renal blood flow (in this as well as in one additional experiment the electromagnetic flowmeter showed irregular and obvious erratic tracings at maximum ischemia Since a normal pattern was restored by retransfusion the failure was probably caused by *constriction of the renal artery)* A slightly greater fall in outer than inner cortical flow at the 70 mm Hg pressure level as revealed by a modest fall in $\overline{OCF}$ $\overline{ICF}$ ratio was not obvious in the other experiment with two step bleeding

Principally similar results were obtained in all seven experiments as summarized in Fig 2—4 Mean outer and inner cortical flow fell in proportion to total renal blood flow (Fig 2) and to each other (Fig 3 and 4) Since the scatter observed in these figures might conseal small but consistent changes in flow distribution the time course of the $\overline{OCF}/\overline{ICF}$ ratio is hown in Fig 5 While the pattern differs from one dog to another there is no consistent change in cortical flow distribution with time

The flow reduction at different electrode sites might differ considerably even in the same cortical layer as evident from Table I and Fig 1 However the gas clearance never fell to zero or to values so low as to suggest complete ischemia with some remaining gas removal by diffusion to circulated areas To test whether hemor rhagic hypotension increased the heterogeneity of cortical flow observed under con trol conditions the SD between electrodes in the same cortical layer was calculated for each H washout In absolute terms the average SD fell to about 1/4 of control However as shown in Fig 6 the ratio between SD and the corresponding $\overline{OCF}$ or $\overline{ICF}$ (coefficient of variation) showed no consistent change either during bleed or after retransfusion

$\overline{OCF}$ ml/ min g	%	Inner cortical blood flow E ml/ min g	%	E_s ml/ min g	%	$\overline{ICF}$ ml/ min g	%	$\overline{OCF/IC}$
3 47	100	4 15	100	3 77	100	3 96	100	0 88
2 68	77	3 07	74	2 77	74	2 92	74	0 92
2 96	85	3 07	74	3 32	88	3 20	81	0 94
2 92	84	3 46	83	3 61	96	3 54	89	0 83
1 96	57	2 37	57	1 80	48	2 09	53	0 94
1 72	50	2 31	56	2 08	55	2 17	55	0 79
1 73	50	2 18	53	2 30	61	2 24	57	0 77
1 61	46	2 31	56	2 35	62	2 33	58	0 69
1 02	29	1 43	35	1 15	31	1 29	33	0 79
1 01	29	0 88	21	1 15	31	1 02	26	0 99
2 63	76	2 44	59	2 96	79	2 70	68	0 97

$\overline{AP}$ mean arterial pressure RBF total renal blood flow E flow at 5 different electrode sites $\overline{OCF}$ and $\overline{ICF}$ mean flow in outer and inner cortex

The slow clearance of H gas during hemorrhage with half times of one minute or more should permit recording of rapid changes in local flow during washout Convincing evidence of such flow changes was not observed in any of the 245 desaturation curves recorded during hemorrhagic hypotension indicating a rather constant minute to minute flow pattern

Renal cortical vascular resistance showed a fairly uniform pattern in all experiments In the early phase of hemorrhagic hypotension RBF $\overline{OCF}$ and $\overline{ICF}$ fell proportionately less than $\overline{AP}$ both with rapid or slow bleeding to 50 mm and with bleeding to 70 mm Hg indicating a fall in vascular resistance Then after 15—30 min of hypotension calculated resistance began to increase passed control level after 30—90 min and reached values of 130—500 % of control after two hours hypotension

As evident from Fig 2 retransfusion did not completely restore cortical flow in any experiment

Discussion

The main findings in the present study were the fairly proportional reduction of inner and outer cortical blood flow during hemorrhagic hypotension and the lack of evidence for patchy or segmental ischemia This conclusion contrasts sharply with the results of several other investigators using other methods for flow measurement In evaluating the results it seems appropriate therefore to pay considerable attention to methodological problems

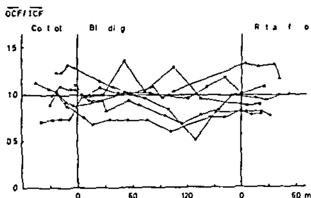


Fig 5 Time course of $\overline{OCF}$/$\overline{ICF}$ ratio in experiments

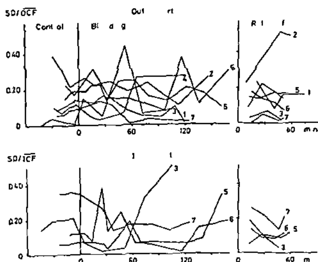


Fig 6 The standard deviat on of local flow at individual electrode sites in outer and inner cortex d vided by the corresponding mean flo Figures on curves ndicate dif ferent experiments

Cortical flow under control conditions Only few attempts ha e been made to measure local cortical flow ith H technique probably because the first studies by Aukland Bower and Berliner (1964) indicated that more than 2/3 of electrodes im planted in the cortex gave multiexponential desaturation curves with problematic interpretation in terms of local blood flow However with further technical refine ment of the method Levning (1971) obtained monoexponential desaturation curves from most electrodes and a good correlation to total renal blood flow This was con firmed in the present study which also showed an excellent reproducibility with a coefficient of variation of only 3 % between consecutive measurements However flow differed considerably between electrode sites within the same cortical layer giving a coefficient of variation of 18 % during control conditions This scatter greater than previously observed in the myocardium with the same technique (Aukland *et al* 1967) suggesting somewhat more heterogeneous flow i

cortex than in the myocardium The alternative explanation that the presence of the electrode should cause more disturbance to local flow in the kidney than in the heart can be neither excluded nor confirmed by the present experiments

The average $\overline{OCF}/\overline{ICF}$ of 0 97 gives obviously no evidence for consistent difference between control flow in deep and superficial layers of the cortex The considerable scatter (0 74 to 1 29) might nevertheless suggest real differences between animals However since the scatter between electrodes in outer and inner cortex was of the same magnitude as between electrodes within the same cortical layer the different $\overline{OCF}/\overline{ICF}$ ratios might well be fortuitous The present experiments thus gave no indication of any flow difference between outer and inner cortex during control conditions

The absolute blood flow averaging 3 47 and 3 61 ml/min×g in outer and inner cortex respectively, is in good agreement with values previously reported by Löyning (1971) with the same technique (3 26 and 3 45) Whether these flow values really represent mean flow in undisturbed cortex (tissue not in close vicinity to electrodes) is difficult to assess due to lack of suitable methods for comparison A rough indication can be given by total renal blood flow divided by kidney weight but a number of uncertainties must be taken into consideration

1) Calibration of the electromagnetic flowmeter probe may probably be off with ±10 % even when calibrated directly on femoral arteries

2) Cortical weight is unknown The present use of the weight of the whole kidney after dissecting out the electrodes may possibly underestimate the weight of the average cortex Organ weight may be reduced by more than 30 % by this procedure as a result of fluid loss from the kidney

3) It is not known to what extent the juxtamedullary circulation will participate in removing H from the juxtamedullary cortex If the washout effect here is zero the calculated cortical flow might well be 20 % too high

Since the 2 last mentioned sources of error might give systematically too high values the average local cortical flow/total flow ratio of 0 67 may not be too far off On the other hand it can not be excluded that insertion and presence of the electrode may reduce blood flow and gas clearance in the tissue immediately surounding the electrode tip

A much higher cortical flow 4—9 ml/min×g is usually obtained by external counting of Kr^{85} or Xe^{133} washout assuming the fastest component (I) of the decomposed washout curve to represent cortical flow (Thorburn *et al* 1963 Carriere *et al* 1966 Truniger Rosen and Oken 1966 Logan *et al* 1971) It is obvious however that this component representing only 50—60 % of kidney volume does not include the whole cortex which anatomically constitutes at least 70 % of the dog kidney (Ullrich 1959) The proposition that the second fastest component (II) which by the inherent mechanics of the peeling off method is at most 1/3 to 1/2 as fast should represent the inner part of cortex together with outer medulla disagrees with the present finding and that of Wolgast (1968) of approximately equal flow in the deep and superficial cortical layers It seems rather more likely that the peeling

off method displays a fairly homogeneous cortical flow as two virtual compartments one relatively large with rate constant exceeding mean cortical flow and one smaller with much lower rate constant Similarly Slotkoff *et al* (1971) comparing their results with those of microsphere distribution concluded that it was not possible to match the first and second components of the xenon washout with any definite anatomical region

Since local gas washout in the cortex presumably is determined by peritubular capillary flow the present results are difficult to compare with microsphere measurements which obviously represent flow through glomeruli Microsphere distribution indicating higher flow in the outer cortex (McNay and Abe 1970 Katz *et al* 1971 Slotkoff *et al* 1971) where glomerular density is highest (Horster Kemler and Valtin 1971) is therefore not necessarily incompatible with the present finding of equal gas washout rates in the various layers This also applies to the similar results obtained by the glomerular basement membrane antibody method recently introduced by Wallin Rector and Seldin (1971)

Measurement of local red cell transit times in various layers of the cortex has been reported by Wolgast (1968) In agreement with the present results he found no difference between flow in deep and superficial layers of the cortex

Cortical blood flow during hemorrhagic hypotension The reduction of renal vascular resistance observed in the initial phase of bleeding followed by steadily increasing values during maintained hypotension agrees well with previous observation by Jirka *et al* (1961) and Aukland and Wolgast (1968) It seems likely that this pattern is determined by an initial autoregulatory response which is gradually overpowered by increasing nervous or humoral vasoconstrictor activity In both phases inner and outer cortical flow varied proportionally and in proportion to total RBF indicating the resistance changes to take place simultaneously and to the same extent in both cortical layers Furthermore totally ischemic areas were never uncountered and the scatter between flow at various electrode sites in the cortex did not increase significantly even when calculated in per cent of actual flow Thus cortical flow remained practically as homogeneous during hemorrhagic hypotension as under control conditions

These conclusions disagree with several previous studies Using Kr^{85} washout technique Carriere *et al* (1966) found that hemorrhagic hypotension produced marked distortion of the various components of the desaturation curves Initially the decay of the fastest component was maintained or even increased but its corresponding tissue volume decreased markedly *Pari passu* the volume represented by the second component (II) increased whereas the flow rate was relatively well maintained On the basis of Kr^{8} autoradiograms these changes were interpreted to mean that a decreasing proportion of the cortex was perfused at normal rate and that the flow in the remaining part mostly superficial layers was reduced to levels corresponding to that of the outer medulla where flow should be preserved fairly unchanged or even increased Similar results have been reported by several other inve using the same technique with washout of Xe^{133} (Truniger Rosen and (

Grandchamps *et al* 1971) and Kr[8] (Carriere and Daigneault 1970) whereas Logan *et al* (1971) who did find considerable reduction of the rate constants of both component I and II ended up with the same conclusion The finding of maintained medullary flow in several of these studies disagrees with the marked reduction observed by locally measured gas washout (H_2 and Kr[8]) and prolongation of medullary transit time for red cells (Aukland and Wolgast 1968) and albumin (Kramer and Deetjen quoted by Kramer 1962) Furthermore the present experiments also failed to confirm the occurrence of outer cortical or patchy ischemia

A possible explanation of this discrepancy was recently offered by Logan *et al* (1971) on the basis of microsphere distribution studies In fair agreement with Xe[133] studies they found that hemorrhagic hypotension reduced the $\overline{OCF}/\overline{ICF}$ ratio when microspheres were injected through catheters introduced through the femoral or brachial artery However ligation and cannulation of one carotid artery (as had been used in the study of Aukland and Wolgast (1968)) brought about a similar reduction in $\overline{OCF}/\overline{ICF}$ and from this control situation bleeding caused no further redistribution It should be noted however that the basis for this conclusion was considerable higher $\overline{ICF}$ (about 60 % but of borderline statistical significance) in animals with carotid occlusion in spite of lower arterial pressure than in control animals implying lower vascular resistance in the inner cortex. Being in the opposite direction of generally accepted baroreceptor response these observations are difficult to accept as reasonable explanation for the finding of proportional reduction of medullary and total renal blood flow In any case the explanation offered by Logan *et al* (1971) does not apply to the homogeneous reduction of cortical flow observed in the present study in which the carotid arteries were left untouched in all experiments Furthermore in unpublished studies we have also failed to find any effect of carotid occlusion on cortical flow distribution

As an attempt to explain the absence of selective ischemia at any electrode site in the outer cortex one might immagine that the presence of the electrode could reduce vascular responsiveness in surrounding tissue thus preventing excessive nervous or humoral vasoconstriction However the parallelity of local and total vascular resistance both during the initial dilatation and the subsequent vasoconstriction seems quite incompatible with this theory

Differences in experimental animals or experimental design must also be considered The present dogs were normally hydrated and 200 ml saline was infused before bleeding was started Unfortunately comparable data are not given in most other studies but it is not unlikely that a routine preoperative withdrawal of food and water might influence the renal vascular response to bleeding

Also the handling of the kidney might be of importance Ofstad Willassen and Egenberg (personal communication) recently found that in dogs with intact kidneys the distribution of microspheres in the renal cortex did not change during hemorrhagic hypotension in good agreement with the present study However when bleeding was performed after the kidney had been rather heavily manipulated the microsphere distribution indicated greater fall in blood flow in outer than in inner cortex

However this observation does not explain the redistribution observed by X^{133} or Kr^{85} methods which do not involve excessive handling of the kidney and it seems therefore most likely that the discrepancy is of methodological origin

An obvious advantage of the hydrogen washout method is that gas clearance is measured locally obviating the need for compartment analysis and matching of the various *compartments* with anatomical regions in the kidney

References

AUKLAND Effect of adrenaline noradrenaline angiotensin and renal nerve stimulation on intrarenal distribution of blood flow in dogs *Acta physiol scand* 1968 *72* 498—509

AUKLAND K B F BOWER and R W BERLINER Measurement of local blood flow with hydrogen gas *Circulat Res* 1964 *14* 164—187

AUKLAND K F KIIL J KJEKSHUS and G SEMB Local myocardial blood flow measured by hydrogen polarography distribution and effect of hypoxia *Acta physiol scand* 1967 *70* 99—111

AUKLAND K and M WOLGAST Effect of hemorrhage and retransfusion on intrarenal distribution of blood flow in dogs *J clin Invest* 1968 *47* 488—501

CARRIERE S and B DAIGNEAULT Effect of retransfusion after hemorrhagic hypotension on intrarenal distribution of blood flow in dogs *J clin Invest* 1970 *49* 2205—2217

CARRIERE S G D THORBURN C C C O MORCHOE and A C BARGER Intrarenal distribution of blood flow in dogs during hemorrhagic hypotension *Circulat Res* 1966 *19* 167—179

GRANDCHAMP A G AYER and B TRUNIGER Pathogenesis of redistribution of intrarenal blood flow in hemorrhagic hypotension *Europ J clin Invest* 1971 *1* 271—276

HERD J A and A C BARGER Simplified technique for chronic catheterization of blood vessels *J appl Physiol* 1964 *19* 791—792

HORSTER M B J KEMLER and H VALTIN Intracortical distribution of number and volume of glomeruli during postnatal maturation in the dog *J clin Invest* 1971 *50* 796—800

JIRKA J V GANZ V FENCL J H CORT and R TRAVNICEK Measurement of renal blood flow in the intact kidney by local thermodilution during haemorrhagic hypotension *Lancet* 1961 2 692—693

KATZ M A R C BLANTZ F C RECTOR JR and D W SELDIN Measurement of intrarenal blood flow I Analysis of microsphere method *Amer J Physiol* 1971 *220* 1903—1913

KRAMER K Renal failure in shock *Shock pathogenesis and therapy* Ed K D Bock Springer Verlag Berlin 1962 *134—144*

LOGAN A P JOSE G EISNER L LILIENFIELS and L SLOTKOFF Intracortical distribution of renal blood flow in hemorrhagic shock in dogs *Circulat Res* 1971 *29* 257—265

LOYNING E W Effect of reduced perfusion pressure on intrarenal distribution of blood flow in dogs *Acta physiol scand* 1971 *83* 191—202

MCNAY J L and Y ABE Pressure dependent heterogeneity of renal cortical blood flow in dogs *Circulat Res* 1970 *27* 571—587

SLOTKOFF L M A LOGAN P JOSE J D AVELLA and G M EISNER Microsphere measurement of intrarenal circulation of the dog *Circulat Res* 1971 *28* 158—166

SMITH H W N FINKELSTEIN L ALIMINOSA B CRAWFORD and M GRABER The renal clearances of substituted hippuric acid derivatives and other aromatic acids in dog and man *J clin Invest* 1945 *24* 388—404

THORBURN G D H H KOPALD J A HERD M HOLLENBERG C C C O MORCHOE and A C BARGER Intrarenal distribution of nutrient blood flow determined with krypton[85] in the unanesthetized dog *Circulat Res* 1963 *13* 290—307

TRUETA R J A E BARCLAY P M DANIEL K J FRANKLIN and M L PRICHARD *Studies of the renal circulation* Blackwell Scientific Publications Ltd Oxford 1947

TRUNIGER B S M ROSEN and D E OKEN Renale Hämodynamik und hämorrhagische Hypotension *Klin Wschr* 1966 *44* 857—862

ULLRICH K J Das Nierenmark Struktur Stoffwechsel und Funktion *Ergebn Physiol* 1959 *50* 433—489

WALLIN J D F C RECTOR JR and D W SELDIN Measurement of intrarenal plasma flow with antiglomerular basement membrane antibody *Amer J Physiol* 1971 *221* 1621—1628

WOLGAST M Studies on the regional renal blood flow with P^{32} labelled red cells and small beta sensitive semiconductor detectors *Acta physiol scand* 1968 *Suppl 313* 1—109

Acta physiol scand 1973 87 526—534
From the Department of Physiology University of Goteborg Sweden

Intestinal Vascular Responses to 5 Hydroxytryptamine

By

BJORN BIBER JOHN FARA and OVE LUNDGREN

Received 21 July 1972

Abstract

BIBER B J FARA and O LUNDGREN *Intestinal vascular responses to 5 hydroxytrypt amine* Acta physiol scand 1973 87 526—534

The *in vivo* and *in vitro* effects of 5 hydroxytryptamine (5 HT) were investigated on the mesenteric vasculature of the cat. Close i a injections (10 μg) or infusions (10—15 μg/min) in to the superior mesenteric artery of chloralose anesthetized cats generally induced an intestinal vasodilatation of 60—150 % above control which was unaltered by α or β adrenergic or cholinergic receptor blockade and which after its development was unaffected by intestinal motility responses Following nerve blockade by tetrodotoxin the vasodilator response to 5 HT was changed to vasoconstriction During 5 HT infusions of 20—50 μg/min intestinal blood flow and intestinal capillary filtration coefficient (CFC) measured plethysmographical ly increased significantly above control while at infusions of 10—15 μg/min 5 HT increased CFC without increasing intestinal blood flow In *in vitro* studies on strips or segments of mesenteric arteries and veins from the cat and rat, 5 HT constantly increased isometric tension in all vessels studied The response was unaltered by adrenergic or cholinergic receptor blockade and was not related to passive tension applied to the vessels nor to the presence or absence of noradrenaline in the bath The divergent *in vivo* and *in vitro* response are discussed in terms of the following possible mechanisms 1 A differential effect of 5 HT with constric tion of large vessels and dilatations of smaller ones 2 A direct constrictor effect on the vascular smooth muscle via specific serotonic receptors as well as an indirect dilator effect perhaps mediated through local nervous pathways

The widespread occurrence of 5 hydroxytryptamine (5 HT) within the gastroin testinal tract of vertebrates including man (Erspamer 1966 Gershon and Ross 1966 Vialli 1966 Penttila and Lempinen 1968) suggests a physiological role in the intestine and has prompted studies concerning its function in the regulation of intestinal motility and blood flow Generally 5 HT causes excitation of intestinal smooth muscle (Erspamer 1966) and stimulates peristalsis when introduced into the intestinal lumen (Bulbring and Lin 1958 Bulbring and Crema 1958 1959 a) or when given intraarterially (Schlesinger *et al* 1959 Haverback *et al* 1957) Applied to the intestinal serosa on the other hand 5 HT inhibits peristalsis (Kosterlitz and Robinsson 1957 Bulbring and Lin 1958 Bulbring and Crema 1958) It has also been demonstrated (Bulbring and Lin 1958 Bulbring and Crema 1959 b Burks and Long 1966) that 5 HT is released into the intestinal lumen and blood stream secondary to peristalsis or increased intraluminal pressure (For reviews se Bulbring *et al* 1970 and Erspamer 1966)

The vascular effects of 5 HT vary between different regional vascular beds and among species Vasoconstrictor responses are reported on large arteries of the dog s front and hind limb (Haddy Gordon and Emanuel 1959 McCubbin Kaneko and Page 1962) as well as on monkey cerebral arteries (Karlsberg Elliot and Adams 1963) rabbit aortic strips (Wurzel 1966) and rat mesenteric venules (Zweifach 1964) On the other hand vasodilator responses have been demonstrated in small arteries of the limb (Haddy Gordon and Emanuel 1959 Wellens and Wauters 1966) and in mesenteric (McCubbin Kaneko and Page 1962 Texter *et al* 1964) and coronary (Hashimoto, Kumakura and Igarashi 1963) circulations in the dog as well as in muscle of the human forarm (Walsh 1967) Thus one cannot generalize concerning 5 HT's vascular effects and its precise role in the regulation of blood flow often remains obscure

In view of the number of studies demonstrating the presence of large amounts of serotonin in the intestinal wall and others describing its contribution to the control of G I motility there are hardly any reports concerning its effects on the mesenteric blood flow This study was therefore undertaken to investigate the *in vivo* and *in vitro* effects of 5 HT on the mesenteric vasculature of the cat

Methods

A In vivo experiments Experiments were performed on 14 cats of both sexes weighing 2 0—3 6 kg The cats deprived of food for 24 h were anesthetized with chloralose i (50—60 mg/kg b w) after ether induction

The operative procedures were similar to those of other studies from this laboratory previously described in detail (B ber *et al* 1971) A proximal jejunal segment weighing 15—25 g was isolated but left *in situ* while the rest of the intestine was extirpated Venous outflow was recorded by means of an optical drop recorder operating an ordinate writer The left and right splanchnic nerves were sectioned and the nerves running along the superior mesenteric artery were also cut Arterial blood pressure was monitored from the left femoral artery *via* a Statham pressure transducer (P23 AC) Blood pressure and flow were recorded on a Grass polygraph I a. injections and infusions were given into a small branch of the superior mesenteric artery

Via a soft plastic cannula placed in the int stinal lumen motility was continuously measured by a Statham low pressure tran ducer (P23 BC) with both ends of the segment tied off

For measurements of intestinal olume and cap llary filtration coeff cient (CFC) the jejunal preparation was placed *in s tu* into a plethysmograph as earl er described (Folkow *t al* 1963 Haglund and Lundgren 1972)

B In vitro experiments Th e studies rep esent experiments don on 13 cat arteries 5 cat veins 4 rat arteries and 9 rat portal vein p eparation Mesenter c blood vessels we e obtained from cats anesthet zed with ether and chloralose and f om rats sacrified by a blow on the neck 5—15 mm long sections of cat mesenteric arcuate or distribut on arteries and veins (outside diameter 0 5—1 mm) and rat superior mesenteric arteries and portal eins were carefully dis ected free and placed into a Krebs solution bath of the following compos tion in mmol/liter

NaCl 122 KCl 4 73 CaCl 2 49 $MgCl_2$ 1 19 $NaHCO_3$ 15 5 KH_2PO_4 1 19 glucose 11 5

Arteries f om the cat and rat were eithe mounted as 4—6 mm long segments or the segments were ut into spiral trips 4—8 mm long one end b ing secured to a steel rod in a 50 ml organ bath the other to a fo ce d placement transduce (G ass FT 03) connected to a Grass polygraph to re ord isometric tension A passi e tension ranging between 200—800 dyn was applied to these arterial vessels The organ bath contained Krebs solution maintained at 37 C and aerated with a gas mixture of 95 % O_2 and 5 % CO_2 the pH being 7 35

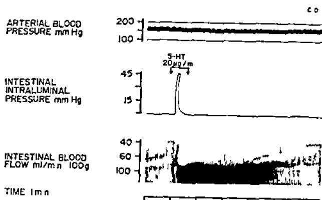


Fig 1 The effect of intraarterial injection of 10 μg 5 hydroxy tryptamine (5 HT) on arterial pressure intestinal intraluminal pressure and venous outflow showing the latency magnitude, and duration of the induced vasodilation

Rat portal and cat veins were similarly mounted as longitudinal segments 4—10 mm long Mechanical responses were recorded isometrically as with the arteries and at a passive tension of 200—500 dyn

2 or 3 different vessel segments could be simultaneously studied in the bath After mounting with the passive tensions a 1 h period was allowed before drugs were added

C Drugs The following drugs were used noradrenaline (Nor exadrine® Astra) phenoxy benzamine (Dibenzyline® Smith Kline and French) propranolol (Inderal® ICI) tetrodotoxin (Calbiochem AG) 5 hydroxytryptamine creatinine sulphate (May and Baker) atropine sulphate and isopropylnoradrenaline sulphate.

Results

A In vivo experiments

Blood flow In 12 cats i a injections of 10 μg 5 HT induced intestinal blood flow increases of 60—150 per cent beyond control appearing within 15 s and reaching maximum within 30 s (Fig 1) and with return to control within another 2—5 min Increasing the 5 HT amount above 10 μg resulted in more prolonged vaso dilator responses (5—10 min) without enhancing the peak flow beyond that caused by 10 μg Vascular tachyphylaxis to 5 HT was not observed with these often re peated single injections

In some experiments the 5 HT vasodilatation was preceded by a transient flow decrease lasting 15—30 s (Fig 1) and in two cats either no blood flow response or a maintained 3—5 min flow reduction was observed Here however obvious rhythmic contractions and intraluminal pressure increases were recorded in phase with the transient flow reduction Thus these apparently diverging effects on blood flow may in some cases be secondary to 5 HT induced motility changes

I a 5 HT infusions (10—50 μg/min) usually induced dilatator responses compar able in size to those induced by single injections of 10 μg but now maintained for 5—15 min Subsequently a gradual return to control occurred despite continuous or even increased 5 HT infusions perhaps representing tachyphylaxis

In no case did i a 5 HT affect arterial blood pressure and therefore could not in this way indirectly influence intestinal blood flow

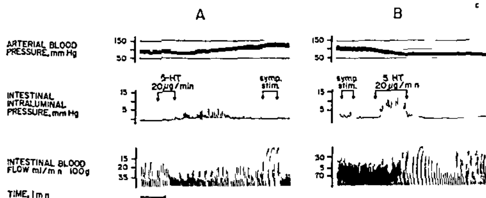


Fig 2 Comparison of the intestinal vascular response to 5 HT infusion (20 µg/min) before (A) and after (B) intraarterial injection of 6 µg tetrodotoxin illustrating the vasoconstrictor response after nerve blockade

Cholinergic blockade (atropine 0 5—1 0 mg/kg i v) and blockade of the adrenergic α receptors (phenoxybenzamine 2 mg/kg i v) and β receptors (propranolol 1 mg/kg i v) did not alter the blood flow response to i a 5 HT as compared to control The effectiveness of the adrenergic blockades was tested by isopropylnoradrenaline and electrical stimulation of local vasoconstrictor nerves

In 5 expts after close i a infusion of tetrodotoxin (6—10 µg) the vasodilator responses to 5 HT administration were abolished as well as the vasoconstrictor response to sympathetic nerve stimulation while vasodilatation was still elicited by i a isopropylnoradrenaline In 3 of the 5 expts 5 HT elicited instead a pronounced and longlasting vasoconstriction after tetrodotoxin (Fig 2) Within 10—20 min the usual responses to 5 HT and sympathetic nerve stimulation were reestablished

2 *Capillary filtration coefficient* During control situations capillary filtration coefficient (CFC) of the intestine ranged from about 0 070 ml/min × 100 g tissue × mm Hg at a blood flow of 20 ml/min × 100 g up to 0 115 when control blood flow was about 60 ml During graded 5 HT infusions intestinal CFC increased significantly ($p < 0\ 001$) beyond the respective control values and usually about 50 per cent (Fig 3) Furthermore without increasing intestinal blood flow 5 HT infusion at 10—15 µg/min clearly increased CFC and the infusion rate had to be raised to 20—50 µg/min to increase both blood flow and CFC

3 *Motility* The first i a injections of 5 HT during an experiment sometimes induced immediate rhythmic contractions of the intestine lasting 15—30 s (Fig 1) This motility increase recorded as intraluminal pressure could also be easily observed visually and appeared to consist of both circular and longitudinal contractions When present this motility response was only seen after the first 5 HT injection while the vasodilator response to 5 HT occurred repeatedly and w

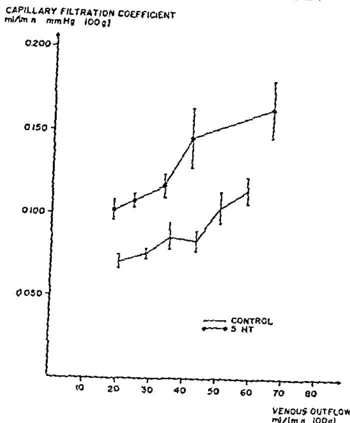


Fig 3 Grouped data representing the relationship between capillary filtration coefficient CFC and venous outflow during control and during 5 HT infusion Nine cats were used for 8 CFC determinations during a range of control blood flow levels and 44 determinations during graded 5 HT infusions of 10—50 µg/min Linear regression analyses of the blood flow versus CFC data using the method of least squares gave the following curves control $y = 0.001\ x + 0.04$ 5 HT $y = 0.001\ x + 0.078$ A t test showed a significant difference between the two curves $p < 0.001$

therefore *per se* independent of the motility response The fading motility response may represent a tachyphylaxis of the visceral smooth muscle to 5 HT which in dogs develops quite rapidly (Haverback *et al* 1957)

B In vitro experiments

5 HT added in concentrations in the bath of 2.5×10^{-6}—25×10^{-6} µg/ml consistantly increased isometric tension in all vessels studied (Fig 4) Within five s after the addition of 5 HT tension rapidly increased to reach a peak In most vessels tension then returned gradually towards control even though 5 HT remained in the bath but several vessels maintained a plateau tension for 5—10 min The tension developed was directly related to the 5 HT concentration and in no case 5 HT reduced the tension below that of the control situation Qualitatively similar responses were observed among the various sizes and types of vessels used but

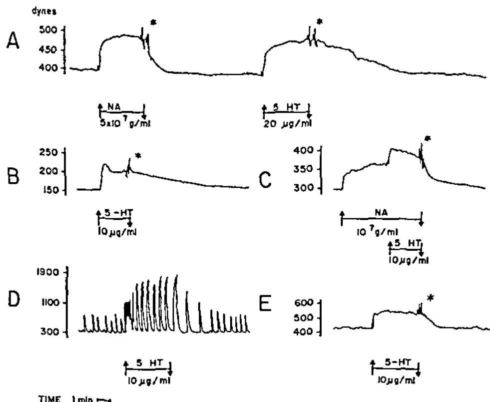


Fig 4 Tension recordings of blood vessels studied *in vitro* showing the responses to 5 hydroxytryptamine (5 HT) and noradrenaline (NA) Concentrations are expressed in wt/volume of bath ★ indicates mechanical artifacts when the bath is rinsed A Comparison of the response to 5 HT and NA in cat mesenteric arcuate artery strip mounted with 400 dyn passive tension B Cat mesenteric arcuate artery segment illustrating often observed initial tension increase which returns towards control during 5 HT exposure period C Record showing the further increase in tension evoked when 5 HT was added during a NA induced tension response *in cat mesenteric arcuate vein segment* D *Tension recording of rat portal vein* illustrating the normal rhythmicity and the increase in contraction frequency and tension during 5 HT exposure F Effect of 5 HT on rat mesenteric artery strip

quantitative comparisons between them were difficult to establish vessels below 500 µm outside diameter could not be studied for technical reasons

In 6 expts the response to 5 HT was studied during exposure to noradrenaline 1×10^{-6}—1×10 g/ml In both cat and rat arteries and veins 5 HT further increased the tension beyond that produced by noradrenaline Isopropylnoradrenaline (1—5×10 g/ml) added to the bath during a noradrenaline or a 5 HT induced tension response decreased the tension toward control levels When isopropylnoradrenaline but not 5 HT was removed from the bath tension again increased toward the previously induced level Furthermore in the rat portal vein preparation isopropylnoradrenaline decreased the amplitude and frequency of the sp

taneous resting activity whereas 5 HT consistently increased both amplitude and frequency (Fig 4)

These effects of 5 HT were not affected by α adrenergic (phenoxybenzamine 1×10^{-8} g/ml) by β adrenergic (propranolol 1×10^{-7} g/ml) or by cholinergic receptor blockade (atropine, 1×10^{-6} g/ml)

Discussion

The present study demonstrates that 5 hydroxytryptamine usually produces intestinal vasodilatation when infused or injected i a in the cat. On the other hand 5 HT increases tension in different vascular smooth muscles studied *in vitro* These two observations are obviously contradictory but may be explained by one of the following mechanisms

1 A differential effect of 5 HT with constriction of large vessels and dilatation of the smaller ones 2 A direct constrictor effect on the vascular smooth muscle via specific serotonin receptors as well as an indirect dilator effect perhaps mediated through local nervous pathways

Ad 1 Previous studies have shown 5 HT to produce either vasoconstriction or vasodilatation within various vascular beds and these effects have been ascribed to differential responses of large *versus* small vessels Haddy *et al* (1959) considered the effects of serotonin on the dog forelimb to be a dilatation of the smallest pre capillary vessels and a constriction of the large ones A similar interpretation was given for the effects of 5 HT on the dog hind limb and mesenteric circulations by McCubbin *et al* (1962) and these authors concluded that the vasodilator effect of HT was mediated through adrenergic vasodilator receptors This is clearly not the case in the current experiments on the cat mesenteric vasculature for the dilator response to 5 HT is unaffected by complete β adrenergic blockade as also shown by Walsh (1967) on the human forearm Additionally in the *in vitro* experiments on isolated vessels the response to 5 HT was unaltered by both α and β adrenergic blocking drugs and also by atropine suggesting a mediation via a specific 5 HT receptor mechanism

In agreement with the *in vivo* studies of Haddy *et al* (1959) McCubbin *et al* (1962) and Texter *et al* 1964 the present *in vitro* experiments on large mesenteric arteries and veins support the idea that 5 HT is a pure constrictor of larger vessels Also when added to a noradrenaline induced tension increase 5 HT further enhanced tension in the large vessels similar to the responses obtained during neurogenic vasoconstriction in the dog hindlimb (McCubbin *et al* 1962) This would mean that the intestinal vasodilator effects of 5 HT occur at a more peripheral level relaxing the most distal precapillary resistance and sphincter sections pronounced enough as to result in a net dilator response of the intestinal vascular bed

While the *in vitro* method does not allow a study of the smallest blood vessels present results concerning overall resistance and capillary filtration suggest a dilator

action of 5 HT of the smallest precapillary vessels CFC increasing significantly at all flow levels This may however also reflect a changed capillary permeability since at lower 5 HT concentrations CFC increased despite an unchanged flow resistance

Ad 2 The hypothesis that the intestinal vascular effects of 5 HT are comprised of both a direct vasoconstrictor action and an indirect vasodilator one is supported by the tetrodotoxin experiments Nerve blockade with this drug reverses the *in vivo* vascular effect of 5 HT from vasodilatation to vasoconstriction indicating that an intact intrinsic nervous function may be essential for the vasodilator response If so the 5 HT influence on such a local nervous mechanism would override the direct vasoconstrictor effects of 5 HT resulting in a net vasodilator response As the intestine was extrinsically denervated in these experiments and the intestinal vasodilator response to 5 HT was unaffected by phenoxybenzamine propranolol or atropine the hypothetical nervous mechanism would be intrinsic non adrenergic and non cholinergic Furthermore the vasodilator action of this suggested nervous pathway would be independent of changes in intestinal motility since there was no consistent relationship between the changes in blood flow and in motility

Thus whether the action of 5 HT is a direct one on the smooth muscles of the smallest vessels or mediated via a local nervous system or a combination of both cannot be determined on the basis of the current experiments However 5 HT is abundantly present in the enterochromaffin cells of the mucosa (Vialli 1966) and small amounts of 5 HT have also been found around the terminal axons of the plexus myentericus (Gershon and Ross 1966) Thus its release and action under appropriate stimuli would greatly influence intestinal blood flow A further study investigating the release of 5 HT and its role as a vasoactive mediator in the regulation of the mesenteric circulation is currently in progress

The authors thank Mrs Eva Bengtsson Mrs Dagny Johansson and Mrs Siv Samuels on for excellent technical assistance This research was supported in part by the Swedish Medical Research Council (No B72 14X 2855 03 and partly No B72 14X 16 08A) and by the Faculty of Medicine University of Goteborg J Fara was supported by a National Institutes of Health Fellowship IFO HE50664 01 from the Heart and Lung Institute

References

BIBER B O LUNDGREN and J SVANVIK Studies on the intestinal vasodilatation observed after mechanical stimulation of the mucosa of the gut *Acta phys l s and* 1971 82 177—190
BURKS T F and J P LONG 5 Hydroxytryptamine release into dog intestinal vasculature *Amer J Physiol* 1966 211 619—625
BULBRING E and A CREMA Observations concerning the action of 5 hydroxytryptamine on the peristaltic reflex *Brit J Pharmacol* 1958 13 444—457
BULBRING E and R C Y LIN The effect of intraluminal application of 5 hydroxytryptamine and 5 hydroxytryptophan on peristalsis the local production of 5 HT and its release in relation to intraluminal pressure and propulsive activity *J Physiol* (Lond) 1958 *140* 381—407
BULBRING E and A CREMA The action of 5 hydroxytryptamine 5 hydroxytryptophan and reserpine on intestinal peristalsis in anaesthetized guinea pig *J Physiol* (Lond) 1959 a *146* 29—53
BULBRING E and A CREMA The release of 5 hydroxytryptamine in relation to pressure exerted on the intestinal mucosa *J Physiol* (Lond) 1959 b *146* 18—28

BULBRING E A T BRADING A W JONES and T TOMITA (Ed) *Smooth Muscle* Edward Arnold Publishers Ltd London 1970
ERSPAMER V Occurrence of indolealkylamines in nature In *Handbook of Experimental Pharmacology* 1966 XIX 132—181
FOLKOW B O LUNDGREN and I WALLENTIN Studies on the relationship between flow resistance capillary filtration coefficient and regional blood volume in the intestine of the cat *Acta physiol scand* 1963 *57* 270—283
GERSHON M D and L L ROSS Localization of sites of 5 hydroxytryptamine storage and metabolism by radioautography *J Physiol* (Lond) 1966 *186* 477—490
HADDY F J P GORDON and D A EMANUEL The influence of tone upon response of small and large vessels to serotonin *Circulat Res* 1959 *7* 123—130
HAGLUND U and O LUNDGREN Reactions within consecutive vascular sections of the small intestine of the cat during prolonged hypotension *Acta physiol scand* 1972 *84* 151—163
HASHIMOTO K S KUMAKURA and S IGARASHI Pharmacological features of coronary vascular vessels *Jap Circulat (En) J* 1963 *27* 149—153
HAVERBACK B J C A M HOGBEN N C MORAN and L T TERRY Effects of serotonin (5 hydroxytryptamine) and related compounds on gastric secretion and intestinal motility in the dog *Gastroenterology* 1957 *32* 1058—1065
KARLSBERG P H W ELLIOTT and J E ADAMS Effect of various pharmacologic agents on cerebral arteries *Neurology* 1963 *13* 772—778
KOSTERLITZ H W and J A ROBINSON Inhibition of the peristaltic reflex of the isolated guinea pig ileum *J Physiol* (Lond) 1957 *136* 249—262
MCCUBBIN J W Y KANEKO and I H PAGE Inhibition of neurogenic vasoconstriction by serotonin Vasodilator action of serotonin *Circulat Res* 1962 *11* 74—83
PENTTILA A and M LEMPINEN Enterochromaffin cells and 5 hydroxytryptamine in the human intestinal tract *Gastroenterology* 1968 *54* 375—381
SCHLEISENGER M H F W SMITH J H PERTH and CH M LEWIS Studies of colon contractility in dogs I Description of method and effect of parasympathomimetic agents and serotonin *J clin Invest* 1959 *38* 2119—2130
TEXTER E C C CHOW S L MERILL H C LAURETA and E D FROHLICH Direct effects of vasoactive agents on segmental resistance of the mesenteric and portal circulation *J Lab clin Med* 1964 *64* 624—633
VIALLI M Histology of the enterochromaffin system In *Handbook of Experimental Pharmacology* 1966 XIX 1—65
WALSH J A Antagonism by methysergide of vascular effects of 5 hydroxytryptamine in man *Brit J Pharmacol* 1967 *30* 518—530
WELLENS D and E WAUTER Norepinephrine induced reflex vasodilation and vascular effects of serotonin *Arch int Pharmacodyn* 1966 *164* 140—149
WENTZEL M Serotonin receptor in rabbit artery *Amer J Physiol* 1966 *211* 1424—1428
ZWEIFACH B W The behavior of the vascular barrier during tissue injury *Bibl Anat* 1964 *4* 21—31

Acta physiol scand 1973 87 535—548

From the Institute of Neurophysiology University of Oslo Norway

Two Types of Lateral Line Afferents in the Eel (Anguilla anguilla)

By

EGIL ALNÆS

Received 24 July 1972

Abstract

ALNÆS E *Two types of lateral line afferents in the eel* (Anguilla anguilla) Acta physiol scand 1973 *87* 535—548

The spontaneous and evoked discharges of eel lateral line sensory organs have been investigated Each afferent nerve fibre has a well defined punctate receptive field corresponding to a single canal organ No axons were found to innervate more than one of these All afferents pass through the Ramus lat dors NX to the medulla oblongata No peripheral unidirectional synapses exist in the nerve Afferent axons are myelinated and belong to a large calibre (mode 12 μm) and a small calibre (mode 3 μm) axonal population in the nerve The nerve contains about 800 myelinated axons at the level of its medullary entry subserving approximately 100 sensory organs In both groups rates of spontaneous discharge range from 0 c/s—50 c/s Both groups include extremely mechano-sensitive units and less sensitive units The main difference between the slowly and rapidly conducting units is the more regular spontaneous firing pattern of the former group

The lateral line organs found in all primary aquatic vertebrates share phylogenetic and ontogenetic origins with the sensory organs of the labyrinth (for reviews of the literature see Dijkgraaf 1963 Cahn 1967 and Flock 1971 a b) The hair cells of the lateral line sensory organs are morphologically and functionally similar to the hair cells of the acoustico vestibular systems being sensitive to mechanical displacement of their cilia by shearing forces from an overlying gelatinous cupula (Flock 1965 1967 *1971 a*) *Accordingly the lateral line system constitutes a linearly distributed* highly specialized mechano-receptive system However its functional significance and mode of operation is essentially unknown (Roberts 1972) and may probably be different in various species

One possible approach to the understanding of its function is to analyse the signal transmission from primary afferent fibres to higher order neurones This however requires a rather detailed knowledge of the organization of the lateral line receptors The present paper contributes such information for the lateral line system of the eel

The spontaneous activity of single afferent axons in the japanese eel (*Anguilla japonica*) was studied by Katsuki *et al* (1951 a b c 1952 a b) These worker

found evidence for two different types of receptor endings supplied by thick and thin nerve fibres, respectively. The former were described as silent when not specifically activated. They had high mechanical thresholds and adapted rapidly. The latter were characterized by high frequency resting discharges. They had low mechanical thresholds and adapted slowly. Dijkgraaf (1963) questioned this interpretation noting the absence of such afferent duality in other lateral line systems. In this paper a modified description of the two types of lateral line afferents is presented.

Material and Methods

Fresh water acclimatized eels (*Anguilla anguilla*) of 250—450 g b.w. and 45—60 cm length were used for all experiments. In initial experiments the fish was anesthetized by immersion in a 0.1 solution of MSS 222 in fresh water and fixed in a specially designed holder. The gills were perfused with ordinary tap water (4—10 C) and the fish regularly sprinkled to prevent drying of the skin and to cool the animal. The temperature at the depth of the lateral line nerve was never found to exceed 16 C at any distance from the head. A high spinalization and destruction of the entire distal spinal cord was performed as vigorous body movements returned within 30 min after the termination of MSS 222 anesthesia. The lateral line nerves on both sides of the trunk were exposed at one or more sites at varying distances (8—0 cm) from the operculum. Particular care was taken to dissect the nerve free without exerting stretch or causing hemorrhage. The nerve on one side of the body was placed in a bipolar platinum L shaped electrode immersed in cooled mineral oil an sewn in under the skin. The distal 2/3 of the body was rotated so that a mineral oil pool could be made for the exposed nerve on the opposite side of the body as well as giving ready access to the whole lateral line canal on that side. The nerve was placed on bipolar *en passage* stimulation and/or registration platinum electrodes or placed *en passage* on a small dissection board. In the latter cases the neural sheath was carefully dissected away and a small subdivision of the nerve isolated by use of fine steel needles and glass hooks. Spontaneous sensory action potentials could be picked up in the tal nerve filament as described by Katsuki *et al.* (1951 a). In most fishes the nerve was left act and a craniotomy performed over the 4th ventricle. The dura covering the cerebellum nd medulla oblongata was dissected away and the cerebellum gently lifted forward on a glass hook to expose the intracranial course of the Ramus lat. post. N. X and its termination in the crista cerebellaris region in the lateral dorsal wall of the 4th ventricle (Herrick 1899, Herser 1901). Single unit action potentials were recorded extracellularly by microelectrode penetration f this part of the nerve. In this region it is possible to assess the circulatory state of the fish by bserving the blood flow in the cerebrospinal vessel. Experiments were terminated if the blood flow deteriorated. Curarization (Tubocurarine 0.1 mg/kg) was necessary to abolish spontaneous head and neck movements. 3 M NaCl micropipette of 3—10 M Ω resistance were used throughout the experiment.

The signals were fed through a cathode follower into a Tektronix 122 preamplifier and an audioamplifier and photographed from a Tektronix 565 4 beam oscilloscope by a Grass camera or processed on line by a Nuclear Chicago 7100 averaging computer. In other experiments the signals were recorded on magnetic tape by an AC tape recorder for later processing on a Nord 1 computer. A comprehensive program system for digital analysis of nervous activity was developed.

Nerve stimulation pulses of 0.1 ms duration were delivered from 2 stimulators through a summing amplifier. The sensory organs were stimulated by gentle stroking by glass rods or small water jets from 0.5 mm polyethylene tubes. The tubes could be affixed to any part of the eel holder thus providing localized adequate stimulation in the manner described by Katsuki *et al.* (1951 b). Further, any particular sensory organ could be mechanically stimulated at precisely timed intervals to electrical nerve stimulation by a blunt glass rod coupled to an electromagnetic vibrator (Katsuki *et al.* 1951 b) or electrically stimulated in the manner described by Katsuki *et al.* (1951 b) and Murray (1956) by direct current pulses (10—100 ms) delivered through the overlying skin by a Ringer agar filled polyethylene tube connected to a DC-stimulator.

Cortland teleost Ringer solution was used throughout the experiments (Wolf 1963).

Sudanese black stained sections of the lateral line nerve were prepared from 10 formalin fixed specimens.

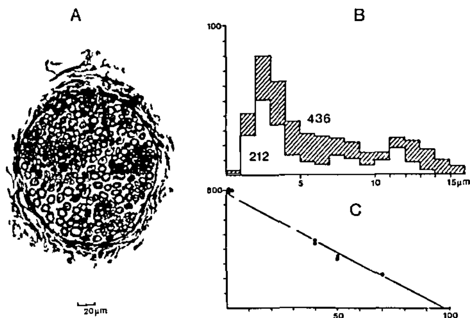


Fig 1 A Cross section of posterior lateral line nerve at the level of the 40th sensory organ from the medulla oblongata Sudan black staining 436 myelinated axons B Axon calibre histograms Hatched columns From section shown in A Empty columns same nerve at level of the 75th sensory organ from the medulla 212 myelinated axons C Number of axons counted in 5 fishes (ordinate) plotted as function of distance from the medulla measured in number of sensory organs on the abscissa

Results

I Anatomy of lateral line nerve

The trunk lateral line nerve in *Anguilla anguilla* originates from sensory organs of the canal type (Katsuki 1952 a) situated along both sides of the body from the operculum to the tail In order to determine the total input to the central nervous system from the posterior lateral line the number of nerve fibres was counted in different cross sections of the nerve (Fig 1 A) The total number of nerve fibres was surprisingly constant in different eels At its intracranial level the nerve contained 797 802 and 803 nerve fibres in three different animals of varying size The distribution of canal openings and hence sensory organs is regular along the lateral line The number of sensory organs varied from 99 to 105 in the present material giving a constant distance between sensory organs of about 5 mm in a medium sized fish The number of nerve fibres decreased regularly with distance along the nerve (Fig 1 C) Apparently each canal organ is exclusively innervated by a fixed number of private axons With a total of 100 canal organs and about 800 axons this gives about 8 nerve fibres to each sensory organ

The axonal diametres of the lateral line nerve vary between 1.5 and 2 (Fig 1 A) and a calibre histogram reveals a bimodal distribution (Fig 1 B)

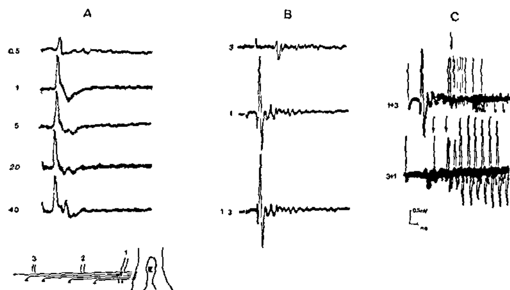


Fig 2 A Compound action potentials recorded *intracranially (1) from the cut end of* R lat dors N V 1—3 superimposed sweeps *En passage* nerve stimulation 8 cm distally (2) at varying stimulation intensities 1 c/s B Electrode numbers as in A Superimposed sweeps registration *en passage* at (2) 1+3 Antidromic volley set up from (1) completely occludes orthodromic volley from (3) (Note *identical ripple form in lower 2 records*) C Superimposed sweeps as in B at various relative delays Observe that stimulation artifacts are retouched (1+3) No occlusion at stimulus delays longer than stimulation timing marked by arrow (3+1) Orthodromic volley from (3) partially occludes antidromic volley from (1) at stimulation delays falling between arrow *Lateral line distal to* (3) 18 cm Lateral line proximal to (3) 17 cm

thick fibre group exhibits a mode at about 12 μm and the thin fibre group one at about 3 μm In analogy with the findings in other sensory systems it is reasonable to *expect that the bimodality of the nerve fibres reflects functional differences between* the two groups of fibres

Supporting and some additional data were obtained by recording the compound action potential of the lateral line nerve

Fig 2 A shows the compound action potential recorded by a bipolar platinum electrode from the intracranially cut end of the trunk lateral line nerve The nerve was stimulated *en passage* by a similar electrode situated 8 cm distally Axon conduction velocities in this fish ranged from 35 m/s (threshold 0 5 V) to approximately 9 m/s *(30 V being a supramaximal stimulus intensity for all* visible components of the neurogram) The nerve evidently contains two major fibre groups with conduction velocities of *ca* 35—15 m/s and 15—10 m/s respectively All fast conducting fibres *followed stimulation frequencies* >*100 c/s There was a moderate reduction* of amplitude in the slower component at this frequency

Fig 2 B (3) shows the supramaximal orthodromic nerve volley recorded *en passage* at electrode 2 distally to the operculum The late ripple in this *response is* produced by the passage of slowly propagating axon potentials across the electrode

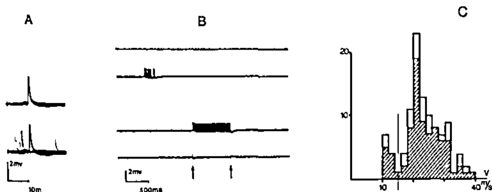


Fig 3 A Single afferent unit recorded extracellularly in the intracranial portion of R lat dors N N Nerve stimulated 20 cm distally The unit is spontaneously active Shown at supra threshold and threshold stimulation intensities B From top to bottom Spontaneous activity Mechanically induced activity (a water jet at skin overlying sensory organ) DC stimulation (between arrows) Two different current intensities C Histogram of unit conduction velocities Hatched columns Spontaneously active units Empty columns Not spontaneously active units

and was remarkably constant in its appearance (superimposed sweeps) B (1) shows the corresponding antidromic volley set up by stimulation of the intracranial portion of the nerve In B (1+3) all peripheral orthodromic activity is occluded distally to the recording electrode the two lower records being virtually identical This was confirmed by computer subtraction of the averaged nerve responses No other path ways therefore exist nor do afferent nerve signals pass through unidirectional synapses in the nerve In Fig 2 C (1+3) the same experiment is shown super imposed at various delays Only stimulation (arrow indicates enhanced stimulation artifact) after the passage (+abs refractory period) of the antidromic volley across stimulation electrode 3 can set up an orthodromic volley from this site C (3+1) shows the antidromic volley (1) preceded by the orthodromic volley (3) Partial occlusion in this experiment occurs when timing of antidromic stimulation falls be tween the arrows Only fibres coming from sensory organs distally to stimulation electrode 3 are occluded *i e* approximately 50 % in this fish of 45 cm trunk length (18 *vs* 17 cm)

II Single unit activity

As reported by Katsuki *et al* (1951 a b) some lateral line nerve fibres showed a background activity in the absence of intentional lateral line stimulation whereas other were silent The silent units were detected by repeated peripheral *en passage* stimulation during exploration of the nerve The frequency of spontaneous activity varied between 0 and 50 c/s In fishes where only proximal holder fastening was used and the distal 2/3 of the fish was left freely submerged in the tank silent units were observed to remain inactive at various stationary body positions

Spontaneous activity was evidently generated in the sensory organs since it always disappeared after sectioning of the nerve

III Statistical description of the spontaneous activity

The firing pattern of neurones may contain information on the mode of generation of the activity (Stein 1965) and could therefore reveal differences between functionally different groups of lateral line sensory units

A systematic analysis of the firing pattern of the units shown in Fig 3 C was conducted in the manner illustrated in Fig 4 A, B

Only units with sensory fields in the distal half of the fish were considered for statistical analysis Thus, all units lying in the proximity of the gills and the heart were excluded With this restriction a marked constancy in firing patterns was observed for most units as illustrated in Fig 4 A, B(b) These computer displays give each successive interval plotted in ms along the ordinate and succession number along the abscissa No marked change in mean frequency or interval range is observed for 2000 successive intervals Sometimes it was possible to observe similar constancies over a period of up to 2 h Stable recording conditions for a period exceeding 5 min were set as a minimum condition for statistical analysis This ensured a minimum interval number of 1000 for all but the very slowly firing units Mean firing frequency ($\bar{x}$) standard deviation (SD) and coefficient of variation (CV = $\overline{SD}/\bar{x}$) were computed and displayed for each unit As seen in Fig 4 A some units were rather regular >90 % of spikes falling in a window of 20 ms width Less regular units were always of the unimodal type shown in Fig 4 B All units were characterized by a rather well defined minimum interval size (x_{min}) In the absence of external stimulation x_{min} was always well above the minimal interval size attained ring maximal external stimulation (>5 ms *cf* Fig 3) *i e* these units did not display the burst firing characteristic of other fish vestibular nerve sensory activity (Enger 1963)

Serial correlation coefficients were computed for interval lags 0—20 ($R_{0\ 1} \ldots {}_{0}$) for all units and displayed in the manner shown in Fig 4 C For all lag values above 0 R was approximately zero *i e* no hidden periodicities of the kinds discussed by Katsuki *et al* (1951 a) occurred This was clearly due to the fact that proximal units were omitted from analysis Units that responded to cardiac contractions and/or respiratory movements were repeatedly found in this region as illustrated in Fig 5 A shows the unit at rest *i e* in the absence of gill perfusion In B the water is turned on and the fish exhibits regular respiratory movements The interval histogram is transformed into a bimodal shape and serial correlation coefficients deviate significantly from zero

In the distally situated units $R_{0\ 1}$ ranged from +0 15 to −0 11 with a mean at +0 06 Units with the higher values were those few regular units in which a slight long term shift in mean firing frequency occurred during the period of registration Coefficients of variation (CV = $\overline{SD}/\bar{x}$) were found to range continuously from 0 09 upwards with a mode at 0 25

Fig 6 A gives a scatter plot of conduction velocity *vs* mean impulse interval for all spontaneously active units with mean intervals <500 ms (some points correspond to more than 1 unit) No obvious correlation is seen between the 2 parametres in

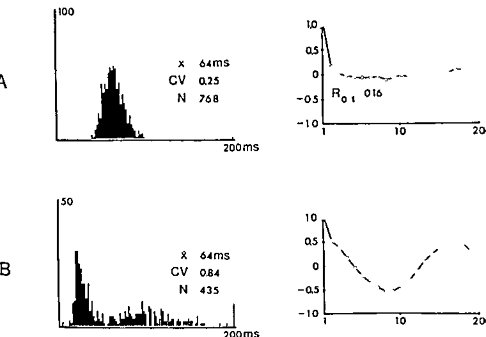


Fig 5 Computer display of activity of proximally situated sensory unit A interval histogram and serial correlation coefficient diagram with no gill movements B do during period of rhythmical respiratory movements

either of the 2 velocity groups in contrast with the finding reported by Katsuki *et al* (1951 a) Nor was a high degree of mechanical sensitivity in any way correlated with frequency of firing or conduction velocity within any of the two fibre calibre groups Similarly it can be seen from the scatter diagram of Fig 6 B that no consistent relationship exists between axonal conduction velocity (and hence fibre diametre) and regularity of firing (measured as coefficient of variation) within the sample of fast conducting units (>14 m/s) Nor is there any correlation between these two parametres in the group of slowly conducting units (<14 m/s) However it can be seen that the two groups differ between themselves in the sense that all slowly conducting units display a rather high degree of regularity (low CV) Thus a receptor duality is apparent in which thin fibre and thick fibre units differ also in regularity of firing although no correlation exists between fibre diametre and receptor characteristics within each of the 2 groups This relationship is brought out more clearly in Fig 6 C in which $\bar{x}$ is plotted against SD for thick fibre units and thin fibre units respectively A marked positive correlation is to be expected between duration of mean interval and its standard deviation throughout a sample of sensory units. This

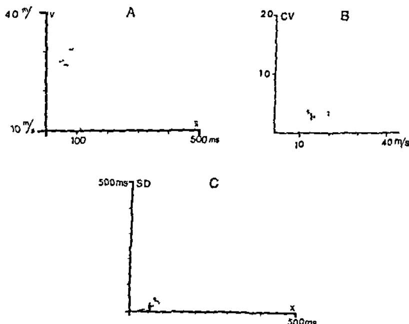


Fig 6 A Scatter diagram of units corresponding to hatched columns in Fig 3 C Mean firing interval (abscissa) plotted against conduction velocity (ordinate) B Scatter diagram of coefficient of variation (ordinate) plotted against conduction velocity (abscissa) for all spontaneously active units Markings as in C C Standard deviation of mean firing interval (ordinate) plotted against mean firing interval (abscissa) for all spontaneously active units Points Units having conduction velocities above 14 m/s Crosses Units having conduction velocities below 14 m/s

is confirmed in the plot in which units with identical degree of firing regularity (CV) would be distributed along a straight line The relationship however is seen to be markedly different in the two samples The sample of slowly conducting units (9 units only) exhibits a lower and more constant SD/$\bar{x}$ ratio As will be dealt with in the discussion the only simple interpretation of this finding is that the mode of generation of the spontaneous activity is different for the two types of fibre

Discussion

The main objective of the present investigation has been to obtain information on the background activity in the eel lateral line system prior to analysis of central nervous mechanisms It has been established that all primary afferents from the trunk lateral line are channeled with no appreciable calibre variation through R post lat N X and that no unidirectional synapses exist peripherally to this portion of the nerve

The bimodal diametre distribution of myelinated nerve fibres found histologically in this study corresponds to the bimodal nerve electrogram The conduction velocity histogram of afferent sensory fibres (Fig 4 C) exhibits a trough at 14 m/s cor

responding to the trough at 7 μm in the axonal diametre histogram (Erlanger and Gasser 1937 data for myelinated frog axons) Thus what in this paper is called thick and thin fibres (from conduction velocity measurements) do in fact correspond to the respective categories as defined histologically

The nerve also contains a number of unmyelinated fibres as do lateral line nerves in other species (Murray 1955 Gorner 1961 Dijkgraaf 1963 Roberts and Ryan 1971) No receptor activity has been discovered for this group and they are commonly thought to serve autonomous functions

The findings of Katsuki et al (1951 b) of localized sensory fields for all afferents were confirmed both directly and indirectly Thus each afferent presumably transmits integrated information on the mechanical shearing forces bearing on an (unknown) number of sensory cells within the same canal sensory organ In essence this principle of localization has been found in all lateral line systems investigated (Dijkgraaf 1963 Cahn 1967) However in lateral line systems of the open type e g that of *Xenopus laevis* the afferent myelinated axons branch off to all receptor groups within the same stitch (Murray 1955) all afferents therefore being influenced by a certain number of disparate sensory cells

Several of the findings reported by Katsuki et al (1951 a b c 1952 a b) have not been confirmed in the present investigation Notably no correlation was found between the spontaneous rate of discharge and fibre diametre nor were low threshold units (mechanical stimulus) restricted to the thinner afferents Also the percentage of non discharging units was not appreciably different in the two fibre groups In the works of Katsuki et al (1951 a b) no electrical stimulation was performed Hence no determinations of fibre conduction velocity were made nor was any clear criterion given for separation of two calibre groups axons being only referred to as thick or thin according to their apparent calibre under the dissecting microscope The diametre histogram thus presented (Katsuki et al 1951 a) does not reveal the bimodal distribution which underlies the separation of fibre grouping in the present study The absence of electrical nerve stimulation would also mask the presence of non discharging single units in the work of these authors The present study reports a rather high (ca 25 %) and similar proportion of such afferents in both calibre groups This finding was clearly not due to mechanical or ischemic damage of sensory organs since as a rule mechanical responses could readily be elicited many silent units having very low mechanical thresholds Nor does it seem reasonable to ascribe this finding to a high temperature or seasonal effect (Katsuki 1951 a) All fishes were kept cooled below 16° C and no significant differences were observed in the period March—October Murray (1955) and Dijkgraaf (1963) have previously questioned the functional differences claimed by Katsuki and coworkers for the two fibre calibre groups However Furukawa and Ishii (1967 a b) reported a somewhat similar duality in primary afferent fibres from a teleostean saccule

The calibre dichotomy observed in the eel lateral line nerve does clearly not constitute a simple grouping of afferent and efferent fibres respectively as is the case in the mammalian ear (Galambos et al 1950 Fex 1962) since the thin fib

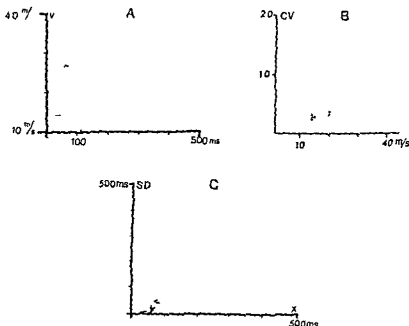


Fig 6 A Scatter diagram of units corresponding to hatched columns in Fig 3 C Mean firing interval (abscissa) plotted against conduction velocity (ordinate) B Scatter diagram of co efficient of variation (ordinate) plotted against conduction velocity (abscissa) for all spontane ously active units Markings as in C C Standard deviation of mean firing interval (ordinate) plotted against mean firing interval (abscissa) for all spontaneously active units Points Units having conduction velocities above 14 m/s Crosses Units having conduction velocities below 14 m/s

is confirmed in the plot in which units with identical degree of firing regularity (CV) would be distributed along a straight line The relationship however is seen to be markedly different in the two samples The sample of slowly conducting units (9 units only) exhibits a lower and more constant SD/$\bar{x}$ ratio As will be dealt with in the discussion the only simple interpretation of this finding is that the mode of generation of the spontaneous activity is different for the two types of fibre

Discussion

The main objective of the present investigation has been to obtain information on the background activity in the eel lateral line system prior to analysis of central nervous mechanisms It has been established that all primary afferents from the trunk lateral line are channeled with no appreciable calibre variation through R post lat. N X and that no unidirectional synapses exist peripherally to this portion of the nerve

The bimodal diametre distribution of myelinated nerve fibres found histologically in this study corresponds to the bimodal nerve electrogram The conduction velocity histogram of afferent sensory fibres (Fig 4 C) exhibits a trough at 14 m/s cor

ferences have not been reported in neuromasts of lateral line systems and could not be investigated by the techniques employed in the present study

Roberts and Ryan (1971) have recently demonstrated two different types of lateral line sensory cells in an elasmobranch (*Scyliorhinus*) (However in this fish the axon diametre histogram is unimodal) The vestibular organs of birds and mammals also contain two different types of hair cells (Wersall 1956) This structural dichotomy has not been reported for hair cells in teleost lateral line sensory organs although the *Lota* axonal calibre distribution is clearly bimodal (Flock 1965) However, in the light of the functional separation of two afferent fibre groups this possibility should be kept in mind in future morphological work on teleost hair cells

The continuous support and help by Dr J K S Jansen is gratefuly acknowledged Computer programming would not have been possible without constructive guidance by Dr O S een and excellent technical assistance was rendered by Mr J Rausandaksel This work was supported by the Norwegian Research Council for Science and the Humanities

References

Alnæs E Peripheral inhibition of eel lateral line receptors as caused by antidromic sensory terminal invasion *Acta physiol scand* 1973a *88* In press

Alnæs E Unit activity of ganglionic and medullary second order neurones in the eel lateral line system *Acta physiol scand* 1973b *88* In press

Cahn P H (ed) *Lateral line detectors* Indiana Univ Press 1967 1—496

Dijkgraaf S The functioning and significance of the lateral line organs *Biol Rev* 1963 *38* 51—105

Enger P S Single unit act ity in the per pheral auditory system of a teleost fish *Acta physiol scand* 1963 Suppl ?10

Erlanger J and H S Gasser *Electrical signs of nervous activity* University of Pennsylvania Press 1937

Fex J Auditory activity in centrifugal and centr petal fibers in cat a study of a feedback system *Acta physiol scand* 1962 Suppl *189*

Flock A Electron microscopic and electrophysiological studies on the lateral line canal o gan *Acta oto laryng* (Stockh) 1965 Suppl *199*

Flock A Ultrastructure and function in the lateral line organs In Cahn (ed) *Lateral Line Detecto s* Indiana Uni Press 1967 163—197

Flock A Sensory transduction n hair cells Pp 396—441 n Loewenste n W R (ed) *Princ ples of R c ptor Physiology* Springe 1971 *I* 1—600

Flock A The lateral l ne organ mechanoreceptors Pp 241— 63 in Hoar W S and Randall D J (eds) *Fish Physiology* Academic Press 1971 I 1—600

Furukawa T and Y I iii Neurophysiol gical studies on hearing n goldfish *Jap J Physiol* 1967 a *17* 57?—588

Furukawa T and Y Ishii N urophysiological studies on hearing in goldfish *J Neurophysiol* 1967 b *30* 1377—1403

Gorner P Beitrag zum Bau und zur Arbe t weise des Se tenorgans von *Xenopus laevis Verh dtsch zool Ges Saa brucken* 1961 193—198

Hama K Some observations on th fine st ucture of the lat ral line organ of the japanese sea eel *Lynco ymba nystromi J cell B ol* 1965 ?4 193—210

Harris G G and A Flock Spontaneous and evoked acti ity f om the *X opus laevis* lateral line In Cahn (ed) 1967 135—161

Harris G G and D C Milne Input output cha acte istics of the lateral line sense organs of *Xenopus laevis J acoust Soc A* 1966 *40* 32—4?

Herrick C J The cranial and f t pinal nerves of *Men dia* A cont ibut on upon the nerve components of the bony fish Ut ca State H spitals P ess 1899 1— 99

Holser G L The neurones and supporting lements of the brain of a selachiar *Neu ol* 1901 *11* 65—175

(< 7 μm) is now proved to contain afferents More will be said about the question of lateral line efferents in a following paper (Alnæs 1972 a) Nor is the dichotomy a reflection of separate groups of high and low threshold sensory organs as maintained by Katsuki *et al* (1951 b) It does, however correspond to two discrete sets of sensory units with different firing characteristics It is worth noticing that the tendency for higher firing regularity observed in the thin fibre group as compared to the thick fibre group does not hold true for fibres of different thicknesses within the two main groups separately

Spontaneous lateral line unit activity in *Xenopus laevis* (Harris and Milne 1966 Harris and Flock 1967 Russell 1968) is rather irregular Most eel lateral line afferents are markedly more regular than those found in *Xenopus laevis* Indeed some fibres display coefficients of variation less than 0 10 Such regularity of firing probably signifies that only one spike initiation site is operative *Xenopus* lateral line afferents on the other hand, branch into several myelinated tram line terminals to different neuromasts Competing spike initiation sites and antidromic invasion of neighbouring branchlets may explain the marked irregularity of firing in this animal, in the manner hypothesized by Harris and Milne (1966) and Harris and Flock (1967) In the eel however, unmyelinated terminal branching occurs only in the immediate vicinity of a single sensory organ (Katsuki *et al* 1952 a) probably rendering such mechanisms unoperative Absence of branchlet spike propagation is not the only prerequisite for regularity however The basic criterion would rather seem to be a sufficiently stable depolarization process at the spike initiation site in which the separate quantal synaptic contributions of the sensory cells are of a small size compared to the fibre threshold and occur at a high spontaneous rate (Stein 1965) This could be ensured by a high degree of synaptic leakage and/or a comparatively long electrotonic distance to the spike triggering zone Both criteria seem to be lacking in saccular afferents which have a markedly irregular spontaneous activity Post synaptic quantal depolarizations as registered by Furukawa and Ishii (1967 a) occur at a low rate and their large size signifies short electrotonic distance The histology of teleost lateral line sensory cells *i e Lyncodymba nystromi* (Hama 1965) *Cnesterodon decemaculatus* and *Fitzroyia lineata* (Trujillo-Cenoz 1961) shows an abundance of presynaptic vesicles and each afferent fibre makes contact with a large (unknown) number of sensory cells According to Katsuki *et al* (1952 a) the thicker afferents in the eel tend to innervate the central portion of the sensory organ with little branching whereas the thinner afferents end on peripheral sensory cells with much branching If the latter mode of arborization means longer electrotonic distance and more frequent synaptic quantal occurrences this difference in terminal architecture may partly explain the higher regularity observed in the group of thin fibres in the present study

In the teleost saccule thick and thin fibres are differently connected as regards directional polarity (Flock 1965) of hair cells Thick (S1) fibres often form synapses with hair cells of both polarities whereas a given thin (S2) fibre only connects to hair cells of the same directional polarity (Furukawa and Ishii 1967 b) Such dif

Acta physiol scand 1973 87 549—556

From the Institute for Experimental Medical Re earch University of Oslo Ullevaal Ho pital Oslo Norway and the Department of Animal Physiology University of Nairobi Kenya

Effect of Physical and Chemical Agents on the Blood Mast Cells (Basophil Leucocytes) of Turtles

By

OLA B REITE[1]

Received 1 August 1972

Abstract

REITE O B *Effect of physical and chemical agents on the blood mast cells (basophil leucocytes) of turtles* Acta physiol scand 1973 *87* 549—556

The histamine releasing effect of several physical and chemical agents on basophil leucocytes was studied in blood from the red eared turtle *Pseudemys scripta elegans* Hypotonic saline low and high pH compound 48/80 and octylamine produced marked histamine release Polymyxin B toluidine blue and protamine seemed to be capable of inducing release of smaller amounts of histamine No significant histamine release was obtained with hypertonic saline d tubocurarine reserpine adrenaline and 5 hydroxytryptamine It is concluded that compared with the tissue mast cells of the rat in which the response to histamine releasing agents has been thoroughl studied the basophil leucocytes of the turtle are considerably more resistant to induced release of h stamine

The resemblance of basophil leucocytes to the tissue mast cells which he had previ ously described prompted Ehrlich (1891) to call them blood mast cells There often seems to be an inverse numeric relationship between these 2 types of cells In man dogs rats and mice tissue mast cells predominate whereas in rabbits and birds the basophil leucocytes are more numerous (Michels 1938 Boseila 1959 Riley 1959) Fresh water turtles (terrapins) have many cells of either type (Michels 1923) Like their mammalian counterparts the tissue mast cells and basophil leucocytes of turtles contain histamine (Reite 1969 1970) The basophil leucocytes apparently are the principal carrier of histamine in blood of both mammals (Graham *et al* 1955 Va lentine *et al* 1955) and turtles (Reite 1970 1972)

Histamine liberators like compound 48/80 degranulate tissue mast cells and release their histamine (Riley 1959 Mota 1966) It has been claimed by some investigators that basophil leucocytes are also degranulated by compound 48/80 (Hunt and Hunt 1958 Marks Sorgen and Ginsburg 1959 Shelley and Juhlin 1962) but others have been unable to confirm this (Levi and Meneghini 1959 Haye and Schneider 1966)

Quantitative studies on histamine release from basophil leucocytes have bee hampered by the scarcity of these cells in most species In the present study bl

[1] P sent add e s Depa tn t / phys ology Uni ers ty of Aarhus Aarhus Den k

KATSUKI Y V MIZUHIRA and S YOSHINO On the endorgan of the acoustico lateralis system of fish *Jap J Physiol* 1952 a *2* 93—102

KATSUKI Y and S YOSHINO Response of the single lateral line nerve fiber to the linearly rising current stimulating the endorgan *Jap J Physiol* 1952 b *2* 219—231

KATSUKI Y S YOSHINO and J CHEN Action currents of the single lateral line nerve fiber of fish I On the spontaneous discharge *Jap J Physiol* 1951 a *1* 87—99

KATSUKI Y S YOSHINO and J CHEN Action currents of the single lateral line nerve fiber of fish II On the discharge due to stimulation *Jap J Physiol* 1951 b *1* 179—194

KATSUKI Y S YOSHINO and J CHEN Neural mechanism of the lateral line organ of fish (Fundamental neural mechanism of sensory organs in general) *Jap J Physiol* 1951 c *1* 264—268

MURRAY R W The lateralis organs and their innervation in *Xenopus laevis* *Quart J micr Sci* 1955 *99* 351—361

MURRAY R W The response of the lateralis organs of *Xenopus laevis* to electrical stimulation by direct current *J Physiol* (Lond) 1956 *134* 408—420

ROBERTS B L Activity of lateral line sense organs in swimming dogfish *J exp Biol* 1972 *56* 105—118

ROBERTS B L and K P RYAN The fine structure of the lateral line sense organs of dogfish *Proc roy Soc B* 1971 *179* 157—169

RUSSELL I J Influence of efferent fibres on a receptor *Nature* (Lond) 1968 *219* 177—178

SAND A The mechanism of the lateral sense organs of fishes *Proc roy Soc B* 1937 *123* 472—495

STEIN R B A theoretical analysis of neuronal variability *Biophys J* 1965 *5* 173—194

TRUJILLO-CENOZ O Electron microscope observations on chemo- and mechano-receptor cells of fishes *Z Zellforsch* 1961 *54* 654—676

WERSALL J Studies on the structure and innervation of the sensory epithelium of the cristae ampullares in the guinea pig *Acta oto laryng* (Stockh) 1956 Suppl *126* 1—85

WOLF K Physiological salines for fresh water teleosts *Progr Fish Cult* 1963 *25* 135—140

Acta physiol scand 1973 87 549—556

From the Institute for Experimental Medical Research University of Oslo Ullevaal Ho pital Oslo Norway and the Department of Animal Physiology University of Nairobi Kenya

Effect of Physical and Chemical Agents on the Blood Mast Cells (Basophil Leucocytes) of Turtles

By

OLA B REITE[1]

Received 1 August 19 2

Abstract

REITE O B *Effect of physical and chemical agents on the blood mast cells (basophil leucocytes) of turtles* Acta physiol scand 1973 87 549—556

The histamine releasing effect of several physical and chemical agents on basophil leucocytes was studied in blood from the red eared turtle *Pseudemys scripta elegans* Hypotonic saline low and high pH compound 48/80 and octylamine produced marked histamine release Polymyxin B toluidine blue and protamine seemed to be cap ble of inducing relea e of smaller amounts of histamine No significant h stamine release was obtained with hypertonic saline d tubocura ine reserpine adrenaline and 5 hydroxytryptamine It is concluded that compared with the tissue mast cells of the rat in which the response to histamine releasing agents has been thoroughly studied the basophil leucocytes of the tu tle are cons derably more esistant to induced release of h stamine

The resemblance of basophil leucocytes to the tissue mast cells which he had previ ously described prompted Ehrlich (1891) to call them blood mast cells There often seems to be an inverse numeric relationship between these 2 types of cells In man dogs rats and mice tissue mast cells predominate whereas in rabbits and birds the basophil leucocytes are more numerous (Michels 1938 Boseila 1959 Riley 1959) Fresh water turtles (terrapins) have many cells of either type (Michels 1923) Like their mammalian counterparts the tissue mast cells and basophil leucocytes of turtles contain histamine (Reite 1969 1970) The basophil leucocytes apparently are the principal carrier of histamine in blood of both mammals (Graham *et al* 1955 Va lentine *et al* 1955) and turtles (Reite 1970 1972)

Histamine liberators like compound 48/80 degranulate tissue mast cells and release their histamine (Riley 1959 Mota 1966) It has been claimed by some investigators that ba ophil leucocytes are also degranulated by compound 48/80 (Hunt and Hunt 1958 Marks Sorgen and Ginsburg 1959 Shelley and Juhlin 1962) but others have been unable to confirm this (Levi and Meneghini 1959 Haye and Schneider 1966)

Quantitative studies on histamine release from basophil leucocytes have been hampered by the scarcity of these cells in most species In the present study I

[1] Present add e s Department of Zoopl ysiology Uni ersit of Aarhus Aarhus D

collected from turtles known to have many basophil leucocytes and accordingly high levels of histamine in blood (Reite 1970) was utilized to overcome this difficulty The histamine releasing effect of compound 48/80 and several other agents was studied

Materials and methods

The experiments were carried out on whole blood from the red eared turtle *Pseudemys scripta elegans* Turtles with body weights of 250—350 g were obtained from an animal dealer in Wisconsin U S A and kept at temperatures of 20—22 C in tanks with shallow water They were fed minced meat and liver once a week Studies were performed during the autumn and early winter The turtles were stunned by a heavy blow on the head and blood was collected from the cut carotid artery into a test tube which had been flushed with a dilute solution of heparin Blood volumes of 8—12 ml were usually obtained Shortly after blood collection 1 ml samples of blood were added on top of 10 ml samples of physiological solution containing the various agents to be tested All experiments were performed in centrifuge tubes The normal exposure time (during which the blood cells were allowed to settle) was 15 min whereupon the suspension was centrifuged for 5 min Histamine content of untreated blood ($\mu g/ml$) as well as total histamine content of sediment and supernatant was determined fluorometrically according to the procedure described by Shore Burkhalter and Cohn (1959) The original method was modified slightly in that citric acid was used instead of hydrochloric acid in the final step of the assay (to stabilize the fluorophore) The histamine content is expressed as μg of the base Fluid samples termed supernatant comprised 10 ml volumes while the remaining fluid was assayed together with the sediment (the latter fractions comprising 1 ml) Blood was exposed to the following agents

Hypo- and hypertonic saline (0—1 8 % NaCl in distilled water) Various values of pH (concentrated HCl or NaOH added to saline) Compound 48/80 (0 01—0 4 mg/ml) Octylamine (0 01—0 4 mg/ml) Polymyxin B sulfate (0 05—1 mg/ml) Toluidine blue (0 25—1 mg/ml) Protamine sulfate (0 025—2 mg/ml) d Tubocurarine chloride (0 05—1 mg/ml) Reserpine (0 05—1 mg/ml) 5 Hydroxytryptamine creatinine sulfate (0 005—0 1 mg/ml) Adrenaline hydrochloride (0 001—0 02 mg/ml)

* Concentrations listed above (expressed as weight of the salts) are the concentrations before addition of the blood sample The effect of compound 48/80 was tested on blood from 4 turtles while other agents were tested on blood from 2 turtles only Experiments concerning effect of changes in osmolarity and pH were carried out in solution of sodium chloride For all other experiments mammalian Ringer solution (buffered) was used Microscopic observations on basophil leucocytes (stained with thionin in 80 % ethyl alcohol) were made before and after exposure to compound 48/80

Results and discussion

Effect of hypo and hypertonic saline

The effect of hypotonic saline is shown in Table I It is noteworthy that no histamine was released until concentrations were reached that also caused hemolysis Hemolysis was in fact evident before significant quantities of histamine could be recovered from the supernatant When blood was added to distilled water more than twice as much histamine remained in the sediment (including the fluid layer on top of the sediment after removal of 10 ml of the supernatant) as that which should be expected if the release was complete In this case when 1 ml blood was added to 10 ml distilled water the final osmolarity was reduced to about one tenth of that found in turtle blood plasma Granules from rat mast cells disrupted by sonic oscillation in a sucrose medium (Lagunoff and Benditt 1963) or by lysis in distilled water (Uvnas 1964) retain most of their histamine but this histamine is rapidly released in the presence of sodium chloride

TABLE I Histamine release from turtle basophil leucocytes in hypotonic solutions (1 ml samples of blood from two turtles number 1 and 3 added to 10 ml samples of dilute solut ons of NaCl in distilled water and the total histamine content of sediment and supernatant determined after centrifugation)

Concentration of NaCl ( )	Turtle no	Histamine content (μg)		Remarks
		Sediment	Supernatant	
0 45	1	16 3	0 2	Swelling of blood cells
0 45	3	7 0	0 1	Swelling of blood cells
0 36	1	17 1	0 2	Swell ng of blood cells
0 36	3	6 4	0 1	Swelling of blood cells
0 27	1	15 9	0 5	Weak hemolys s
0 27	3	6 5	0 2	Weak hemolysis
0 18	1	13 7	1 9	Strong hemolysis
0 18	3	5 8	0 4	Strong hemolysis
0 09	1	9 2	5 8	Strong hemolysis
0 09	3	5 7	1 7	Strong hemolys s
0	1	? 9	13 6	Strong hemolys s
0	3	1 8	4 8	Strong hemolysis

Hypertonic saline (concentrations of NaCl up to 1 8 %) did not release significant amounts of histamine Some histamine was always present in the supernatant (up to 0 2 μg) but this amount was not different from that found in control experiments with physiological saline or Ringer solution

Effect of cha iges in pH

Various hydrogen ion concentrations in the range between pH 3 and pH 11 did not produce histamine release At pH 2 and pH 12 release of considerable amounts of histamine (20—30 % of that pre ent) occurred and at pH 2 hemolysis was also evident Solutions of sodium hydroxide has earlier been reported to degranulate skin mast cells of mice (Takeda 1958)

Effect of compound 48/80

Compound 48/80 had a marked histamine releasing effect and more histamine eemed to be released at concentrations of 0 2—0 3 mg/ml than at higher and lower concentrations (Table II) At the highest concentrations of compound 48/80 there was reason to su pect that this agent might interfere with the assay of histamine in the supernatant The purified extract became partially opaque after addition of NaOH (before addition of *o* phthalaldehyde) and then acquired a pinkish colour when acidified to stabilize the fluorophore after reaction with *o* phthalaldehyde However even at these concentrations of compound 48/80 the amount of histamine recovered from sediment and supernatant corresponded fairly well to the amount found in 1 ml of untreated whole blood collected from the same turtle

Influence of exposure time and temperature on the effect of compound 48/80 was studied in blood from one turtle The results are shown in Table III At 20° C there was no significant difference in the amount of histamine released after exposures of

TABLE II Histamine releasing effect of compound 48/80 on basophil leucocytes Results obtained after incubation of blood from three different turtles (number 5 8 and 9) with various concentrations of 48/80 are listed

Conc of compound 48/80 (mg/ml)	Turtle no	Histamine content (µg)	
		Sediment	Supernatant
0 4	5	16 0	7 4
0 4	8	7 8	11 4
0 4	9	8 5	2 1
0 3	5	11 3	9 9
0 3	8	7 2	12 1
0 3	9	9 4	2 7
0 2	5	10 8	11 0
0 2	8	10 3	9 8
0 2	9	8 9	3 0
0 1	5	16 7	5 5
0 1	8	14 2	8 5
0 1	9	9 7	1 9
0 05	5	18 3	2 4
0 05	8	15 7	3 6
0 05	9	9 8	1 6
0 025	5	18 5	1 1
0 025	8	18 6	1 4
0 025	9	12 0	0 9
0 01	5	22 1	0 3
0 01	8	20 3	0 5
0 01	9	12 1	0 2

5 15 or 40 min but the degree of histamine release was markedly less at 5° C and 35° C than at 20 C Histamine release from rat mast cells induced by compound 48/80 is also affected by temperature (Uvnas 1958)

Microscopic observations of thionin stained smears of normal blood cells and blood cells treated with compound 48/80 indicated that histamine release was accompanied by degranulation of the basophil leucocytes The morphological changes seemed to be similar to those described for tissue mast cells of the rat (Bloom and Haegermark 1965)

Effect of octylamine

Histamine release was apparent at octylamine concentrations of 0 05 mg/ml and at concentration of 0 1 mg/ml and higher most of the histamine was recovered from the supernatant (Table IV) Hemolysis occurred at octylamine concentrations of 0 2 mg/ml and seemed to be complete at concentrations above 0 3 mg/ml It has previously been clearly demonstrated that octylamine causes histamine release from mammalian tissue mast cells (Mota 1959 Bray and Van Arsdel 1961 Uvnas 1961)

Effect of polymyxin B

Polymyxin B interfered with the assay of histamine The extract became partially opaque when made alkaline before addition of *o* phthalaldehyde and the opacity

TABLE III Influence of exposure time and temperature on the histamine releasing effect of compound 48/80 (300 μg/ml) on turtle basophil leucocytes (each value is the mean of two experiments with blood from the same turtle)

Exposure time (min)	Temperature (C)	Histamine content (μg)	
		Sediment	Supernatant
5	20	5 3	9 1
15	20	4 9	8 8
40	20	4 7	8 6
15	5	13 8	1 5
15	35	11 2	1 7

TABLE IV Histamine release from turtle basophil leucocytes incubated with octylamine Results obtained in experiments with blood from 2 turtles (no 6 and 11) are shown

Conc of octylamine (mg/ml)	Turtle no	Histamine content (μg)		Remarks
		Sediment	Supernatant	
0 4	6	2 4	21 4	Strong hemolysis
0 4	11	1 3	10 2	Strong hemolysis
0 3	6	2 7	19 5	Strong hemolysis
0 3	11	1 3	9 9	Strong hemolysis
0 2	6	3 7	18 8	Weak hemolysis
0 2	11	1 2	10 8	Weak hemolysis
0 1	6	4 6	16 9	
0 1	11	1 7	9 0	
0 05	6	19 5	3 1	
0 05	11	8 4	2 6	
0 025	6	22 6	0 4	
0 025	11	11 5	0 7	
0 01	6	21 7	0 3	
0 01	11	11 1	0 2	

persisted after addition of citric acid However the histamine content of the sediment could be determined after several washings with Ringer solution This showed that after exposure to polymyxin B and subsequent washing there was considerably less histamine left in the sediment than after similar washing of blood cells exposed to pure Ringer solution It thus seemed that polymyxin B was able to induce release of histamine from turtle basophil leucocytes In mammals polymyxin B is recognized as a potent liberator of histamine from mast cells of the rat (Parratt and West 1957 Lagunoff and Benditt 1960 Moran Uvnas and Westerholm 1962)

Effect of toluidine blue

Being a commonly used stain for mast cells toluidine blue has been suggested to cause histamine release without degranulation by occupying the binding sites for histamine on the mast cell granules (Smith 1958) The present experiments with concentrations of toluidine blue of 0 25—1 mg/ml indicated that this agent may also

TABLE V Histamine release from turtle basophil leucocytes incubated with protamine The ei was studied in blood from 2 turtles (no 14 and 15)

Co c of protamine (mg/ml)	Turtle no	Histamine content (µg)	
		Sediment	Supernatant
2	14	18 8	0 2
2	15	20 1	0 3
1	14	21 0	0 4
1	15	22 4	0 4
0 5	14	19 3	2 9
0 5	15	19 7	2 4
0 2	14	18 5	2 3
0 2	15	18 9	1 8
0 1	14	17 8	1 5
0 1	15	19 1	1 7
0 05	14	18 7	0 6
0 025	14	19 3	0 3

histamine from turtle basophil leucocytes but it must be emphasized that the latte conclusion is based on determinations of the histamine content of the sediment afte several washings with Ringer olution Presence of toluidine blue otherwise inter fered with the assay of histamine

Effect of protamine

The effect of protamine is shown in Table V It seems that this agent was not a very tent relea er of histamine from turtle basophil leucocytes More histamine was released at intermediate (0 1—0 5 mg/ml) than at higher and lower concentrations which indicate that lack of strong effects can hardly be ascribed to binding of protamine by the heparin used as anticoagulant Histamine releasing effect of protamine on mammalian tissue mast cells has been amply demonstrated (Archer 1959 Bray and Van Arsdel 1961 Smith 1958)

Effect of d tubocurarine reserpine adrenaline and 5 hydroxytryptamine

None of these agents induced release of significant amounts of histamine but some histamine was perhaps released at the highest tested concentrations of 5 hydroxy tryptamine This agent has been reported to produce histamine release in the domestic fowl *Gallus domesticus* (Buñag and Walaszek 1962) Of the other agents d tubocurarine is the only one which has been found to have a marked histamine releasing effect in higher vertebrates (Paton 1956 Selye 1965) Reserpine inter fered with the assay of histamine in the supernatant in the way that the peak of the fluorescence spectrum was found at 500—530 nm instead of at 450—460 nm

General comments

Compared with mammalian tissue mast cells the basophil leucocytes of the turtle seem to be remarkably resistant to induced release of histamine With compound

48/80 the amount of histamine released never exceeded 65 % of that present in the cells and the concentration of histamine liberator necessary to achieve histamine release was about 100 times higher than that shown to release most of the histamine from mast cells of the rat (Bloom and Haegermark 1965) The failure of some investigators to produce degranulation in mammalian basophil leucocytes by compound 48/80 may be due to a difference in sensitivity between the latter cell type and tissue mast cells towards this agent but variations among species should also be borne in mind

The present investigation was supported by the Norwegian Council on Cardiovascular Diseases Compound 48/80 (condensation product of p-methoxyphenethylmethylamine with formaldehyde) was supplied by AB Leo Halsingborg Sweden

References

ARCHER G T The release of histamine from the mast cells of the rat *Austral J exp Biol med Sci* 1959 *37* 383—390

BLOOM G D and O HAEGERMARK A study on morphological changes and histamine release induced by compound 48/80 in rat peritoneal mast cells *Exp Cell Res* 1965 *40* 637—654

BOSEILA A W A *The basophil leucocyte and its relationship to the tissue mast cell* Copenhagen Munksgaard 1959

BRAY R E and P P VAN ARSDEL In vitro histamine release from rat mast cells by chemical and physical agents *Proc Soc exp Biol* (N Y) 1961 *106* 255—259

BUNAG R D and E J WALASZEK The mechanism of serotonin and tryptamine induced vasodepression in the chicken *J Pharmacol exp Ther* 1962 *135* 151—155

EHRLICH P *Farbenanalytische Untersuchungen zur Histologie und Klinik des Blutes* Berlin, Hirschwald 1891

GRAHAM H T O H LOWRY F WHEELWRIGHT M A LENZ and H H PARISH JR Distribution of histamine among leucocytes and platelets. *Blood* 1955 *10* 467—481

HAVE R H and R SCHNEIDER, The difference in behaviour of basophil leucocytes and mast cells towards compound 48/80 *Brit J Pharmacol* 1966 *28* 282—288

HUNT T E and E A HUNT The effect of 48/80 on the blood basophils of cockerels *Anat Rec* 1958 *130* 419—420

LAGUNOFF D and E P BENDITT Mast cell degranulation and histamine release observed in a new in vitro system *J exp Med* 1960 *112* 571—580

LAGUNOFF D and E P BENDITT Proteolytic enzymes of mast cells *Ann N Y Acad Sci* 1963 *103* 185—197

LEVI L and C L MENEGHINI Liberatori di istamina istaminemia e mastociti circolanti in alcune dermatos alle gi he *G ital De m* 1959 *100* 613—621

MARKS B H R SORGEN and H GINSBURG Histamine liberator 48/80 and basophilic leucocytes *Biochem Pharmac l* 1959 2 200—205

MICHELS N A The mast cell in the lower vertebrates *Cellule* 1923 *33* 337—462

MICHELS N A The mast cells In *Handbook of Hematology* ol I edited by H Downey New York Hoeber 1938 pp 231—372

MORAN N C B UVNAS and B WESTERHOLM Release of 5 hydroxytryptamine and histamine from rat mast cells *Acta physiol scand* 1962 *56* 26—41

MOTA I Effect of antigen and octylamine on mast cells and histamine content of sensitized guinea pig tissues *J Physiol* (Lond) 1959 *147* 425—436

MOTA I Release of histamine from mast cells In *Handbuch der experimentellen Pharmakologie* vol XVIII part 1 edited by O Eichler and A Farah Berlin Springer 1966 pp 569—636

PARRATT J R and G B WEST Release of 5 hydroxytryptamine and histamine from tissues of the rat *J Physiol* (Lond) 1957 *137* 179—192

PATON W D M The mechanism of histamine release In *Histamine* Ciba Found Symp London J & A Churchill Ltd 1956 pp 59—73

REITE O B Phylogenetical persistence of the non mast cell histamine stores of the digestive tract a comparison with mast cell histamine *Experientia* (Basel) 1969 *25* 276—277

REITE O B, The evolution of vascular smooth muscle responses to histamine and 5 hydroxytryptamine III Manifestation of dual actions of either amine in reptiles *Acta physiol scand* 1970 *78* 213—231

REITE O B Comparative physiology of histamine *Physiol Rev* 1972 *52* 778—819

RILEY J *The mast cells* Edinburgh E & S Livingstone Ltd 1959

SELYE H *The mast cells* London Butterworths 1965

SHELLEY W B and L JUHLIN In vitro effect of lecithinase A on the cytology of the human basophil *J Lab clin Med* 1962 *60* 589—597

SHORE P A A BURKHALTER and V H COHN JR A method for the fluorometric assay of histamine in tissues *J Pharmacol exp Ther* 1959 *127* 182—186

SMITH D E Nature of the secretory activity of the mast cell *Amer J Physiol* 1958 *193* 573—575

TAKEDA Y On the variation of stainability and of the mast cell granules in inflammatory tissue *Okajimas Folia anat jap* 1958 *31* 63—78

UVNAS B The mechanism of histamine liberation *J Pharm Pharmacol* 1958 *10* 1—13

UVNAS B Mechanism of release from mast cells *Chemotherapia* (Basel) 1961 *3* 137—149

UVNAS B Release processes in mast cells and their activation by injury *Ann N Y Acad Sci* 1964 *116* 880—890

VALENTINE W N J S LAWRENCE M L PEARCE and W S BECK The relationship of the basophil to blood histamine in man *Blood* 1955 *10* 154—159

Acta physiol scand 1972 87 557—566

From the Departments of Psychiatry S t Gorans Hospital and Pharmacology Karolinska Institutet Stockholm Sweden

Effect of Spinal Transection on the Metabolism of 5 Hydroxyindoles Formed in vivo from ³H-Tryptophan in the Rat Spinal Cord

By

JOHAN SCHUBERT

Received 20 September 1972

Abstract

SCHUBERT J *Effect of spinal transection on the metabolism of 5 hydroxyindoles formed in vivo from ³H tryptophan in the rat spinal cord* Acta physiol scand 1973 87 557—566

The nerve impulse flow in 5 hydroxytryptamine (5 HT) neurons descending to the caudal part of the spinal cord of rats was interrupted by a spinal transection At various time intervals after the operation ³H tryptophan was administered i v and the accumulations of labelled 5 HT and 5-hydroxyindoleacetic acid (5 HIAA) in the cord were determined 6—8 days after the section the cord caudal to the lesion was almost depleted of 5-HT and very little ³H 5 HT was formed from the labelled precursor Between 8 and 24 h post lesion the level of 5 HT was increased by 20 below the lesion whereas the accumulation of ³H 5 HT was markedly reduced Immediately after the transection the endogenous 5 HT level as well as the ³H 5 HT accumulation were unchanged in the caudal cord The accumulation of ³H 5-HIAA below the section was reduced at all time intervals after the operation. The specific activity of tryptophan seemed to be increased following nerve section The results indicate that 5-HIAA synthesis is controlled by nerve impulse activity The synthesis of 5 HT was not directly dependent on neuronal activity or tryptophan levels but seemed to be inversely correlated to the endogenous 5 HT level

Most of the 5 hydroxtryptamine (5 HT) in the rat spinal cord is stored in terminals of axons descending from nerve cell bodies in the brain stem (Carlsson *et al* 1964 Dahlstrom and Fuxe 1965) Following a transection of the spinal cord the rate of disappearance of 5 HT after inhibition of tryptophan hydroxylase is reduced caudal to the lesion (Anden Fuxe and Hokfelt 1966) Electrical stimulation of the spinal cord *in vitro* is followed by release of 5 HT into the surrounding media (Anden *et al* 1965) and stimulation of the medulla oblongata *in vivo* causes a reduction of 5 HT in the spinal cord (Dahlstrom *et al* 1965) These results indicate a relation between nerve impulse activity and the rate of 5 HT turnover in the spinal cord Similar effects on the turnover rate of 5 HT in the forebrain are found after acute destruction or stimulation of the medial raphe nuclei (Aghajanian Rosecrans and Sheard 1967 Kostowski *et al* 1968 Sheard and Aghajanian 1968)

Several mechanisms for the regulation of 5 HT synthesis and turnover have been proposed Recently the tissue level of the amino acid precursor tryptophan as a rate limiting factor for the formation of 5 HT has been emphasized (Airaksinen Giacalone and Valzelli 1968 Moir and Eccleston 1968 Grahame Smith 1971 and Tagliamonte *et al* 1971) However few investigators have studied the levels of tryptophan in relation to impulse induced alterations of 5 HT turnover

The aim of the present investigation was to study the effect of nerve transection on synthesis and turnover of 5 HT as well as on levels of tryptophan in order to elucidate regulatory mechanisms for the formation and release of the transmitter The *in vivo* formation of 5 HT and 5 hydroxyindoleacetic acid (5 HIAA) from the precursor ^{3}H tryptophan was determined in the spinal cord at various time intervals following a total transection at the upper thoracic level

Material and Methods

Male Sprague Dawley rats weighing 140—180 g were used The experiments were run at an ambient temperature of 23 C ^{3}H l Tryptophan (4 05—5 17 Ci/mmol generally labelled NEN Chemical GmbH) was dissolved in isotonic saline and administered i v in a volume of 0 5 ml Drug used was probenecid (Astra)

Experimental

The rats were anesthetized with pentobarbital sodium (40 mg/kg i p) When animals were operated only 10 min before ^{3}H tryptophan administration (see below) diethylether was used for anesthesia Following partial laminectomy the spinal cord was transected at the third fourth thoracic segment To ascertain disconnection of spinal cord parts at the point of lesion about 2 mm of the cord was removed Body weight and body temperature were regularly registered after transection

At less than 10 min 12—15 h (acute transection) or 8 days (chronic transection) after the operation ^{3}H tryptophan was rapidly injected or infused during 20 min into a tail vein Animals were killed by decapitation 1 or 3 h following the ^{3}H tryptophan injection or immediately after the infusion The spinal cord cranial and caudal to the lesion were rapidly taken out and cooled on ice In non operated control rats the whole spinal cord was removed and divided into a cranial and caudal portion corresponding to the cord parts obtained from animals subjected to spinal transection

Chemical procedure

Spinal cord regions from 2 rats were pooled weighed and homogenized in 6 ml of ice cold 0 4 N perchloric acid (containing 0 2 % ascorbic acid) After centrifugation of the extracts endogenous and labelled tryptophan 5 HT and 5 HIAA were isolated from the supernatant fluid by chromatography on columns of Amberlite CG 50 type 1 Dowex 50W X4 and Sephadex G 10 (Schubert to be published)

The labelled compounds were determined by dissolving an aliquot of the column eluates in Insta Gel (Packard Instr Co) followed by liquid scintillation counting in a Packard Tri-carb spectrometer with absolute activity analyzer Endogenous tryptophan and 5 HT were determined fluorimetrically according to modifications of the methods described by Hess and Udenfriend (1959) and Bogdanski *et al* (1956) respectively

Results

The contents of tryptophan and 5 HT in the spinal cord at various time intervals after transection

The content of tryptophan below the lesion during the first 48 h after transection was not significantly changed either in comparison to the tryptophan level in the

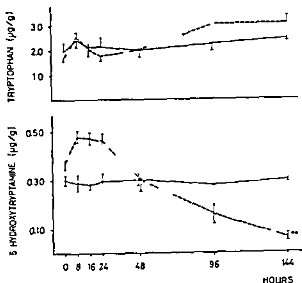


Fig 1 Concentrations of endogenous tryptophan and 5 HT in the cranial (———) and caudal (— — — —) part of the spinal cord at various time intervals after transection Each point represents the mean value ± S E of 4—5 determinations Differs from zero-time group x) $p < 0.05$ xx) $p < 0.01$ xxx) $p < 0.001$

same spinal cord part of controls or compared to the tryptophan concentration above the section (Fig 1) At 4 and 6 days after operation the level of tryptophan caudal to the lesion was significantly increased in relation to that of non operated controls The level of 5 HT in the spinal cord above the section was not altered at any time interval after the operation (Fig 1) Below the section the 5 HT level was possibly significantly increased at 8 and 16 h after operation when compared to the same spinal cord part in control animals When values from the time interval between 8 and 24 h were pooled and compared to the control value the increase was significant ($p < 0.01$) After 24 h the 5 HT level started to decrease and was significantly reduced after 96 h Six days (144 h) after transection the 5 HT concentration was about 15 % of that of non lesioned controls

Animals subjected to a spinal cord transection weighed about 20 % less than controls 6 days after the operation The body temperature was not altered at any time interval after the transection

Effect of a chronic spinal cord transection on accumulation of labelled 5 HT and 5 HIAA formed from ³H tryptophan in the spinal cord

Rats were subjected to a spinal cord transection 8 days before a 20 min constant rate i v infusion of ³H tryptophan Immediately after the infusion the animals were killed and the contents of labelled 5 HT and 5 HIAA as well as the specific activity of tryptophan were determined in the spinal cord above and below the lesion

The accumulations of labelled 5 HT and 5 HIAA in the cord cranial to the section were increased about two-fold compared to those in controls (Fig 2) Cau

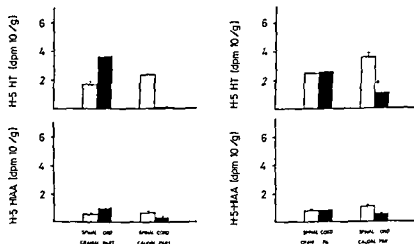


Fig 2 Accumulation of labelled 5 HT and 5 HIAA formed from ^{3}H tryptophan in the cranial and caudal part of the spinal cord 8 days after transection ^{3}H Tryptophan (100 μCi) was infused i v for 20 min and animals were killed immediately thereafter Black columns signify operated animals white columns controls Each column represents the mean value + S E of 6—7 determinations xx) $p < 0\,01$ xxx) $p < 0\,001$

Fig 3 Accumulation of labelled 5 HT and 5 HIAA formed from ^{3}H tryptophan in the cranial and caudal part of the spinal cord 12—15 h after transection ^{3}H Tryptophan (100 μCi) was infused i v for 20 min and animals were killed immediately thereafter Black columns signify operated animals white columns controls Each column represents the mean value $\pm$ S E of 6—8 determinations xxx) $p < 0\,001$

to the lesion the ^{3}H 5 HT and ^{3}H 5 HIAA accumulations were about 5 % and 40 % respectively of the values in the same cord part of non operated controls The specific activity of tryptophan in the caudal part of the cord was possibly significantly higher in operated animals when compared with controls whereas in the cranial part there was no significant difference (Table I)

Effect of an acute spinal cord transection on accumulation of labelled 5 HT and 5 HIAA formed from ^{3}H tryptophan in the spinal cord

Rats were subjected to a spinal cord transection 12—15 h before a 20 min constant rate i v infusion of ^{3}H tryptophan Immediately after the infusion the animals were sacrificed and the contents of labelled 5 HT and 5 HIAA as well as the specific activity of tryptophan were determined in the spinal cord above and below the lesion

In the cranial cord the accumulations of labelled 5 HT and 5 HIAA were not significantly altered by the transection (Fig 3) The accumulation of ^{3}H 5 HT in the cord caudal to the lesion was reduced to about 30 % of the value in non operated control animals and the ^{3}H 5 HIAA accumulation in the same cord part to about 50 % of that in controls The specific activity of tryptophan in the cord parts did not differ between operated and control animals (Table I)

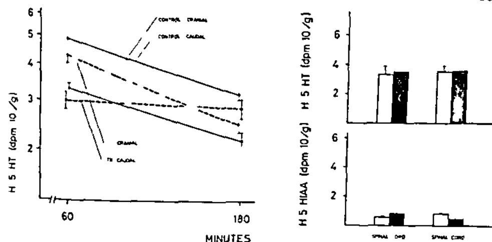


Fig 4 Disappearance of ^{3}H 5-HT formed from ^{3}H tryptophan in the cranial and caudal part of the spinal cord 12—15 h after transection (Tx) ^{3}H Tryptophan (120 μCi) was injected i v and groups of animals were killed 1 or 3 h later Each point represents the mean value ±S E of 4—5 determinations Regression lines were calculated according to the method of least squares The rate constants (k) were

	Spinal cord part	k H 5 HT
Controls	Cranial	0 20±0 04
	Caudal	0 19±0 03
Transected	Cranial	0 24±0 04
	Caudal	0 03±0 04*

Differs from caudal cord of controls ($p < 0\ 01$) Differs from cranial cord of transected animals ($p < 0\ 01$)

Fig 5 Accumulation of labelled 5 HT and 5 HIAA formed from ^{3}H tryptophan in the cranial and caudal part of the spinal cord immediately after transection ^{3}H Tryptophan (100 μCi) was infused i v for 20 min and the animals were killed immediately thereafter Black columns sham operated animals white columns controls Each column represents the mean value ± S E of 6—8 determination x) $p < 0\ 05$

Effect of an acute spinal cord transection on disappearance of ^{3}H 5 HT formed from ^{3}H tryptophan in the spinal cord

Between 12 and 15 h after a spinal cord transection rats were injected i v with ^{3}H tryptophan Groups of animals were killed 60 and 180 min after precursor injection and the contents of labelled tryptophan and 5 HT in the cord above and below the lesion were determined

60 min after ^{3}H tryptophan administration the content of ^{3}H 5 HT above the lesion was significantly higher in comparison with the content in non-operated controls (Fig 4) Below the section the content of labelled 5 HT was lower than in the controls

The mean level of H 5 HT in both spinal cord parts of operated as well as control animals declined during the time interval between 60 and 180 min after injection of the labelled precursor (Fig 4) The rate of decline of ^{3}H 5 HT calculated according to the method of least squares from a first order disappearance of ^{3}H 5 HT

TABLE I The specific activity of tryptophan in the spinal cord following a 20 min i v infusion of ^{3}H tryptophan at various time intervals after a spinal cord transection Figures represent mean value ± S E of 7–8 determinations

Time after transection		Tryptophan specific activity (μCi/μg)	
		Cranial cord	Caudal cord
Immediately	Controls	0 31 ± 0 02	0 25 ± 0 01
	Operated	0 28 ± 0 02	0 29 ± 0 02*
12–15 h	Controls	0 24 ± 0 02	0 25 ± 0 02
	Operated	0 27 ± 0 01	0 27 ± 0 01
8 days	Controls	0 18 ± 0 01	0 16 ± 0 01
	Operated	0 21 ± 0 02	0 19 ± 0 01*

* Differs from control group $p < 0.05$

(Schubert and Sedvall 1972) was significantly reduced below the lesion of operated animals in comparison to the ^{3}H 5 HT disappearance in the cranial part of the same cord and also compared to that in the caudal part of controls The k values for the disappearance of the labelled amine in the cord above the lesion and in the cord parts of control animals did not differ significantly

The specific activity of tryptophan was about the same in all spinal cord regions both at 60 and 180 min respectively after precursor injection

Accumulation of labelled 5 HT and 5 HIAA formed from ^{3}H tryptophan in the spinal cord immediately following a transection

Within 10 min after a spinal transection ^{3}H tryptophan was infused i v at a constant rate during 20 min Immediately after the infusion the animals were killed and the contents of labelled tryptophan 5 HT and 5 HIAA as well as the levels of endogenous tryptophan and 5 HT were determined in the spinal cord above and below the lesion

The accumulations of ^{3}H 5 HT cranial and caudal to the section were about the same as in control animals (Fig 5) The ^{3}H 5 HIAA accumulation was possibly significantly increased above the lesion whereas below the lesion it was possibly significantly reduced when compared to the control value The specific activity of tryptophan was possibly significantly higher in the caudal cord of operated animals whereas in the cranial part it did not differ significantly from the control value (Table I)

The levels of endogenous tryptophan and 5 HT in the cord parts were the same in transected animals as in controls

In a similar experiment probenecid (200 mg/kg) was administered i p to all animals 10 min before the infusion of ^{3}H tryptophan Here the accumulation of ^{3}H 5 HIAA was significantly reduced in the caudal cord of operated animals when compared to non-operated controls (Table II) Above the lesion the ^{3}H 5 HIAA

TABLE II Accumulation of labelled 5 HIAA formed from ³H tryptophan in the cranial and caudal cord of probenecid treated rats immediately after a spinal cord transection Probenecid (200 mg/kg) was administered i p 10 min before a 20 min i v infusion of ³H tryptophan (100 μCi) Animals were killed immediately after the infusion Figures represent mean value $\pm$ S E of 5–6 determinations

	Spinal cord part	³H 5 HIAA dpm 10 g
Controls	Cranial	1 8 ± 0 1
	Caudal	2 1 ± 0 1
Transected	Cranial	1 7 ± 0 1
	Caudal	1 4 ± 0 1*

* Differs from control group $p < 0.01$

accumulation was not altered The specific activities of tryptophan and 5 HT were not significantly changed in any region after the cord transection

Discussion

Intravenous administration of ³H tryptophan is followed by formation of labelled 5 HT and 5 HIAA in the brain and spinal cord (Neff *et al* 1971 Schubert and Sedvall 1972 Schubert to be published) Accumulation of ³H 5 HT in central neurons is dependent on the specific activity of the precursor the rate of 5 HT synthesis and the degree of retention of the newly formed amine by the endogenous stores Accumulation of ³H 5 HIAA is determined by the rate of 5 HT release and catabolism and the transport of 5 HIAA into the circulation Since 5 HT seems to be stored in more than one compartment in 5 HT neurons (Thierry Fekete and Glowinski 1968 Shields and Eccleston 1972) and the degree of equilibration of newly formed ³H 5 HT within these stores is insufficiently known the specific activities of 5 HT and 5 HIAA are of doubtful meaning Thus calculation of the absolute turnover rate of 5 HT from the specific activity in tissue homogenates of accumulated ³H 5 HT or ³H 5 HIAA may be erroneous (Eccleston 1972 Schubert and Sedvall 1972) In the *present study* the accumulation of ³H 5 HT after a short i v infusion of ³H tryptophan was used as an index of the relative synthesis rate of the endogenous amine In order to obtain an estimate of relative 5 HT release and catabolism during the infusion interval the accumulation of ³H 5 HIAA was determined

Histochemical studies have shown that a few days after transection of the spinal cord the 5 HT-containing nerve fibres in the part below the lesion begin to disappear and none or only few persist after 8 days (Carlsson *et al* 1964) In accordance with this the present biochemical findings show that 6—8 days after a spinal cord transection the caudal cord is almost depleted of endogenous 5 HT

while very little ^{3}H 5 HT and a reduced amount of ^{3}H 5 HIAA is formed from the precursor ^{3}H tryptophan in comparison with the accumulations of labelled 5 HT and 5 HIAA in the intact caudal spinal cord (Fig 1 and 2) Thus after this time interval the synthesis and storage of 5 HT seem to be diminished or abolished probably owing to degeneration of 5 HT neurons

The reduction of the ^{3}H 5 HIAA accumulation was not as pronounced as that of ^{3}H 5 HT A corresponding observation was made by Giacalone and Kostowski (1969) who found that one week after a lesion of the midbrain raphe area, where some of the bulbospinal neurons originate the level of 5 HT was significantly reduced in the spinal cord whereas the level of 5 HIAA remained unchanged It is possible that part of the 5 HIAA in the caudal spinal cord originates from serum and cerebrospinal fluid or is released from intact segmental interneurons having a low storage capacity of 5 HT (Clineschmidt Pierce and Lovenberg 1971) However, caution is warranted in interpreting this finding since the ^{3}H 5 HIAA content determined after transection was low and close to the limit of the method

In animals where the spinal cord had been transected for 8 days the accumulations of labelled 5 HT and 5 HIAA respectively in the cord above the lesion were markedly augmented (Fig 2) This increase in 5 HT synthesis and catabolism might be due to a compensatorily increased nerve impulse activity in the intact part of the spinal cord after chronic section

After acute transection of the spinal cord the accumulations of labelled 5 HT and 5 HIAA below the lesion were significantly reduced (Fig 3) indicating that both synthesis and catabolism of 5 HT are diminished as a consequence of the ceased nerve impulse flow Since the disappearance of ^{3}H 5 HT from the caudal cord after prelabelling of the amine stores was reduced in acutely operated animals (Fig 4) it is probable that the diminished ^{3}H 5 HIAA accumulation reflects a reduced release of the endogenous transmitter

The synthesis of 5 HT and 5 HIAA from tryptophan have been shown to be partly dependent on substrate level (Moir and Eccleston 1968 Graham Smith 1971 Fernstrom and Wurtman 1972) It is not likely that the levels of tryptophan plays any part for the reduction of 5 HT synthesis and release found in the present results since the specific activity of the precursor in the spinal cord below transection was unchanged or increased (Table 1) in the presence of reduced accumulations of labelled 5 HT and 5 HIAA The present experiments do not exclude the possibility that the uptake and transport mechanisms of tryptophan in 5 HT neurons might be impaired after nerve section as suggested by Carlsson et al (1972)

The data in Fig 1 demonstrate that the level of endogenous 5 HT increased by about 20 % during the first 24 h post lesion before the nerve fibres had started to degenerate This increase of the concentration of 5 HT after interruption of the impulse flow is analogous to the initial increase in dopamine content after lesion of the nigrostriatal dopamine pathway (Faull and Laverty 1969 Andén et al 1972 Nyback 1972) It suggests a dissociation between synthesis and release of the transmitter and indicates that nerve impulse activity does not directly regulate 5 HT

synthesis In favour of this view is the finding that immediately after nerve transection before the level of endogenous 5 HT increased significantly the accumulation of ^{3}H 5 HT was unaltered whereas the accumulation of ^{3}H 5 HIAA was reduced This indicates that the initial elevation of the level of endogenous 5 HT after ceased impulse activity is due to diminished release and metabolism of the transmitter in the presence of continued synthesis Thus 5 HT release and catabolism is better correlated to nerve impulse activity than 5 HT synthesis

An increased transport of 5 HIAA from the spinal cord cannot account for the decreased ^{3}H 5 HIAA accumulation following interruption of the impulse flow since this decrease was present also after inhibition of 5 HIAA efflux from brain with probenecid That the formation of ^{3}H 5 HIAA was reduced by only 30 % in probenecid treated animals after nerve section indicates that 5 HT is metabolized even in the absence of impulse flow This supports the view that a considerable deamination of 5 HT occurs intraneuronally through a non functional pool (Graham Smith 1971 Bedard Carlsson and Lindqvist 1972)

It seems as if the size of 5 HT pools limits synthesis of the transmitter since the formation of ^{3}H 5 HT appeared to be reciprocally related to the increase of the 5 HT level following interruption of the impulse flow This corresponds to the findings that after monoamine oxidase inhibition neuronal impulses do not influence 5 HT synthesis (Meek and Fuxe 1971) and that there is negative correlation between accumulation of ^{3}H 5 HT formed from intracisternally injected ^{3}H tryptophan and the level of endogenous 5 HT (Macon Sokoloff and Glowinski 1971) Furthermore an inverse relationship between 5 HT levels and neuronal activity in raphe units has been found by Aghajanian (1972)

From the above results it may be concluded that the synthesis of 5 HIAA in the spinal cord is regulated by nerve impulse activity There is no evidence for a direct impulse regulation of 5 HT synthesis However the results argue in favour of an endproduct-control of the synthesis of the amine In addition intra neuronal break down of 5 HT might be regulating the level of 5 HT It is not likely that the tissue level of the precursor tryptophan is related to impulse induced changes of 5 hydroxyindole metabolism

The excellent technical assistance of Miss Johansson and Miss Margareta Saker is gratefully acknowledged The author thanks Mrs Hedi Hedrenius and Mrs Elsa Rylander for preparing the manuscript

The investigation was supported by grants from the Swedish Medical Research Council (project B72 14X 2381 05) the Swedish Foundation for applied medical defense research (project 8 034/71) Svenska Sallskapet for Medicinsk forskning and Karolinska Institutet

References

AGHAJANIAN G K Influence of drugs on the firing of serotonin containing neurons in brain *Fed Proc* 1972 *31* 91—96

AGHAJANIAN G K J A ROSECRANS and M H SHEARD Serotonin release in the forebrain by stimulation of midbrain raphe *Science* 1967 *156* 402—403

AIRAKSINEN M M E GIACALONE and L VALZELLI Hydroxylation of tryptophan in the brain in vivo *J Neurochem* 1968 *15* 55—56

while very little ^3H 5 HT and a reduced amount of ^3H 5 HIAA is formed from the precursor ^3H tryptophan in comparison with the accumulations of labelled 5 HT and 5 HIAA in the intact caudal spinal cord (Fig 1 and 2) Thus after this time interval, the synthesis and storage of 5 HT seem to be diminished or abolished probably owing to degeneration of 5 HT neurons

The reduction of the ^3H 5 HIAA accumulation was not as pronounced as that of H 5 HT A corresponding observation was made by Giacalone and Kostowski (1969) who found that one week after a lesion of the midbrain raphe area where some of the bulbospinal neurons originate the level of 5 HT was significantly reduced in the spinal cord whereas the level of 5 HIAA remained unchanged It is possible that part of the 5 HIAA in the caudal spinal cord originates from serum and cerebrospinal fluid or is released from intact segmental interneurons having a low storage capacity of 5 HT (Chneschmidt Pierce and Lovenberg 1971) However caution is warranted in interpreting this finding since the ^3H 5 HIAA content determined after transection was low and close to the limit of the method

In animals where the spinal cord had been transected for 8 days the accumulations of labelled 5 HT and 5 HIAA respectively in the cord above the lesion were markedly augmented (Fig 2) This increase in 5 HT synthesis and catabolism might be due to a compensatorily increased nerve impulse activity in the intact part of the spinal cord after chronic section

After acute transection of the spinal cord the accumulations of labelled 5 HT and 5 HIAA below the lesion were significantly reduced (Fig 3) indicating that both synthesis and catabolism of 5 HT are diminished as a consequence of the ceased nerve impulse flow Since the disappearance of ^3H 5 HT from the caudal cord after prelabelling of the amine stores was reduced in acutely operated animals (Fig 4) it is probable that the diminished ^3H 5 HIAA accumulation reflects a reduced release of the endogenous transmitter

The synthesis of 5 HT and 5 HIAA from tryptophan have been shown to be partly dependent on substrate level (Moir and Eccleston 1968 Graham Smith 1971 Fernstrom and Wurtman 1972) It is not likely that the levels of tryptophan plays any part for the reduction of 5 HT synthesis and release found in the present results since the specific activity of the precursor in the spinal cord below transection was unchanged or increased (Table I) in the presence of reduced accumulations of labelled 5 HT and 5 HIAA The present experiments do not exclude the possibility that the uptake and transport mechanisms of tryptophan in 5 HT neurons might be impaired after nerve section as suggested by Carlsson *et al* (1972)

The data in Fig 1 demonstrate that the level of endogenous 5 HT increased by about 20 % during the first 24 h post lesion before the nerve fibres had started to degenerate This increase of the concentration of 5 HT after interruption of the impulse flow is analogous to the initial increase in dopamine content after lesion of the nigrostriatal dopamine pathway (Faull and Laverty 1969 Anden *et al* 1972 Nyback 1972) It suggests a dissociation between synthesis and release of the transmitter and indicates that nerve impulse activity does not directly regulate 5 HT

Acta physiol scand 1973 87 567—569

From the Department of Physiology University of Goteborg Sweden

Are There Any Significant Inertial Losses in the Vascular Bed?

By

E ELIASSEN[1] B FOLKOW and B ÖBERG

It is generally assumed that flow resistance in vascular beds is essentially determined by vessel geometry and perfusate viscosity while inertial losses are usually disregarded except when turbulence is considerable However Benis Usami and Chien (1970) recently suggested that a substantial portion of the pressure drop in a vascular bed is after all due to inertial losses known to occur in tube systems showing taperings sharp bends and curves and where localized three-dimensional flows arise They based their claims on results obtained when the dog paw was alternately perfused with two Newtonian fluids of different viscosities If no inertial losses occurred and vascular geometry remained constant the ratio between the flows of the low and high viscosity fluid when measured at identical pressure should then remain constant over the whole pressure range and correspond to the inversed ratio between the *in vitro* viscosities Benis *et al* found however that the flow ratio gradually decreased when perfusate flows increased which was taken to indicate that inertial factors contribute significantly to flow resistance especially at high flow rates

The possibility remains however that the flows of the two perfusates were after all not compared at identical vessel dimensions because of accidental adjustments of vascular tone It seems likely that the more abundant flows obtained with the low viscosity fluid imply a relatively improved tissue nutrition which may favour autoregulatory augmentations of vascular tone Such possible active reductions of vessel dimensions might explain the non constant flow ratio reported by Benis *et al*

To elucidate this problem the experiments of Benis *et al* were in principle repeated in the present study although great precautions were taken to prevent autoregulatory adjustments of vascular dimensions by maintaining the vessels maximally dilated In 12 experiments the calf muscle vascular bed of the cat (Djojosugito *et al* 1969) was alternately perfused with two Newtonian fluids of different viscosities (6 % Macrodex Tyrode solution and 2 % Rheomacrodex Tyrode or 4 % Ficoll Tyrode solution) The viscosities were measured with an Ostwald viscometer Arterial and venous pressures (P_A P_V) were measured with Statham

[1] Present address Department of Physiology University of Bergen Norway

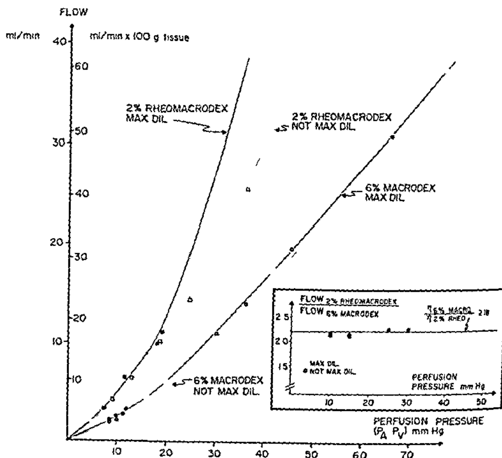


Fig 1 Pressure flow relationships for 6 Macrodex Tyrode and 2 % Rheomacrodex Tyrode solutions obtained when no attempts were made to keep the vascular bed maximally dilated (open symbols) and when maximal dilatation was ensured by *i a* papaverine infusion and intermittent exercise (filled symbols) — The insert shows the ratio between the flow of the two perfusates at different perfusion pressure and flow levels The flow ratio gradually decreases when no maximal dilatation is at hand (open symbols) while it is essentially constant when the vascular smooth muscles remain completely relaxed (filled symbols) and then coincide with the inversed viscosity ratio (η 6 % Macro/η 2 % Rheo)

transducers from branches of the femoral artery and vein Venous outflow was continuously measured by an optical drop recorder ordinate writer unit Pressures and flows were recorded on a Grass Polygraph Maximal vasodilatation was induced either by intermittent exercise (stimulation of the motor fibres to the calf muscles) or/and by *i a* injections of large amounts of vasodilator drugs (papaverine and/or isoprenaline) When a maximal steady state flow was reached at a given pressure head exact flow values were obtained by collecting the outflow in a graduated cylinder

By recording flow at various levels of perfusion pressure pressure flow curves for the two perfusates were constructed both when no deliberate attempts were made to

ensure maximal dilatation (as in the experiments of Benis *et al*) and when maximal vasodilatation was induced

Fig 1 shows such pressure flow relationships for 6 % Macrodex Tyrode and 2 % Rheomacrodex Tyrode obtained in one typical experiment The viscosities of the two fluids were 2 4 cP and 1 1 cP respectively In constructing the curves great care was taken to keep the mean distending pressure $\frac{(P_A+P_V)}{2}$ the same at a given pressure head (P_A-P_V) for the two perfusates in order to avoid passive alterations of vessel geometry As seen flow at a given pressure head is less when maximal vasodilatation was not ensured than in case a maximal dilatation really was at hand particularly evident for the low viscosity fluid at higher perfusion pressures Thus in the absence of vasodilator agents the vascular smooth muscles are still capable of autoregulatory adjustments even during perfusion with such a relatively poor nutrient fluid as oxygenated Dextran Tyrode

The insert in Fig 1 shows the ratio between the flows for the two perfusates measured at identical pressure heads and distending pressures as a function of the perfusion pressure When maximal vasodilatation is not ensured this ratio decreases gradually with increasing pressures and flows as reported by Benis *et al* and by them interpreted as due to increasing inertial losses However when maximal dilatation prevails throughout the flow ratio remains essentially constant even at flow rates above the maximal physiological flows in skeletal muscles (50—70 ml/100 g ×min) This flow ratio also corresponds well with the inversed viscosity ratio for the two perfusates

The present findings strongly suggest that inertial factors play such an insignificant role within the physiological range of blood flows that they can usually be neglected

This investigation was supported by grants from the Swedish Medical Research Council (B73 14X 644 09B B73 14X 16 09B)

References

BENIS A M S USAMI and S CHIEN Effect of hematocrit and inertial losses on pressure flow relations in the isolated handpaw of the dog *Circulat Res* 1970 *27* 1047—1069

DJOJOSUGITO A M B FOLKOW B ÖBERG and S WHITE A comparison of blood viscosity measured *in vitro* and in a vascular bed *Acta physiol scand* 1970 *78* 70—84

Acta physiol scand 1973 87 570—572

From the Departments of Pharmacology and Physiology Karolinska Institutet Stockholm, Sweden

Increased Release of Noradrenaline from Stimulated Guinea Pig Vas Deferens after Indomethacin Treatment

By

Bertil Fredholm and Per Hedqvist

Received 19 January 1973

In recent years it has become increasingly clear that prostaglandins of the E series (PGEs) are potent inhibitors of sympathetic neuroeffector transmission (*cf* Hedqvist 1973) and it has been suggested that endogenous and locally formed PGEs may be of importance as modulators of effector responses to sympathetic nerve activity (*cf* Hedqvist 1973) In the course of a study of mechanisms controlling sympathetic neurotransmission it was observed that indomethacin a potent inhibitor of PG synthesis (Vane 1971) increased noradrenaline (NA) release from the electrically stimulated guinea pig vas deferens

15 male guinea pigs weighing 500—700 g were used for the study The animals were killed by a blow on the head and the vasa deferentia were isolated and incubated for 1—2 h in Tyrode's solution containing 25 µCi/ml ^{3}H l-NA (N E N spec act 6 4 Ci/mmol) They were then carefully rinsed and superfused at 37° C in a 2 ml organ bath at a rate of 1 ml/min with NA free Tyrode The superfusate was collected in 1 ml fractions and analyzed for radioactivity in a Packard Liquid Scintillation Spectrometer Trains of square wave pulses (300—450 pulses 5 Hz 1 ms duration supramaximal voltage) were given at 10—20 min intervals via electrodes in the wall of the bath

Cation exchange column chromatography (amberlite) revealed that intact NA constituted more than 90 [illegible] of the radioactivity retained in the organ and more than 70 % in stimulated superfusate Hedqvist 1973)

In the absence of indomethacin superfusate from stimulated vasa deferentia contained smooth muscle active acid lipid material co chromatographing with PGEs on silica gel plates (Hedqvist and Euler 1972 while superfusate from vasa deferentia pretreated with indomethacin 10 g ml for 0 min contained no such material

Transmural stimulation of the guinea pig vas deferens caused increased and reproducible efflux of radioactivity known to consist mainly of intact ^{3}H NA (Hedqvist 1973) Administration of a small dose of PGE_1 or PGE [illegible] (10 ng/ml) markedly and reversibly depressed the release of NA in respons to this type of stimulation

Infusion of indomethacin (2—10 µg/ml) did not change the resting efflux of radioactivity but was followed after 10—20 min of increased release of ^{3}H NA in response to transmural stimulation (Fig 1) After the infusion of indomethacin was stopped the increased ^{3}H NA overflow response to stimulation was maintained for approximately 15 min whereafter it slowly subsided during the next 30—60 min Infusion of PGEs (10 ng/ml reversed the potentiating effect of indomethacin on

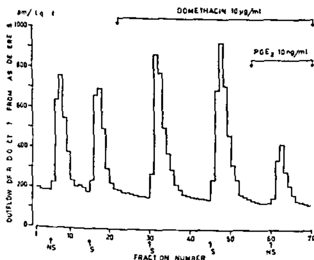


Fig 1 Isolated superfused guinea pig vas deferens loaded with ^{3}H NA Outflow of radioactivity from the organ resting and in response to transmural stimulation (NS) 450 pulses 1 ms 5 Hz. Effect of indomethacin (10 μg/ml) followed by PGE_2 (10 ng/ml)

^{3}H NA release and likewise blocked the effect when given before indomethacin (Fig 1 2)

Comment

Indomethacin is a potent inhibitor of PG synthesis (Vane 1971) and the compound was found to increase NA efflux from the stimulated guinea pig vas deferens Since this effect of indomethacin was completely abolished by small doses of PGE it is

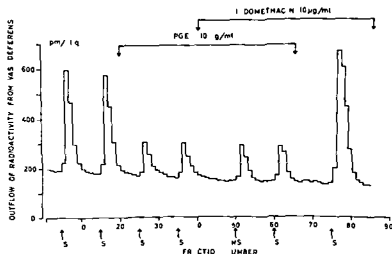


Fig 2 Isolated superfused guinea pig vas deferens loaded with ^{3}H NA Outflow of radioactivity from the organ, resting and in response to transmural stimulation (NS) 450 pulses 1 ms 5 Hz Effect of PGE (10 ng/ml) followed by indomethacin (10 μg/ml)

suggested that indomethacin increases NA release from the sympathetic nerves of the guinea pig vas deferens by means of inhibition of local PG formation The present observations together with the finding that the completely unrelated PG synthesis inhibitor 5 8 11 14 eicosatetraynoic acid increases the release of NA from stimulated rabbit heart cat spleen and guinea pig vas deferens (Samuelsson and Wennmalm 1971 Hedqvist *et al* 1971 Hedqvist 1973) must be considered to strongly support the view that locally formed PGs serve the function to modulate sympathetic neuroeffector transmission

Supported by grants from the Swedish Medical Research Council 14X 3027 3186 and from Stiftelsen Lars Hiertas Minne

References

Hedqvist P Prostaglandin mediated control of sympathetic neuroeffector transmission *Advances in the Biosciences* 1973 9 461—473

Hedqvist P and U S v Euler Prostaglandin controls neuromuscular transmission in guinea pig vas deferens *Nature New Biol* 1972 *236* 113—115

Hedqvist P L Stjärne and A Wennmalm Facilitation of sympathetic neurotransmission in the cat spleen after inhibition of prostaglandin synthesis *Acta physiol scand* 1971 *83* 430—432

Samuelsson B and A Wennmalm Increased nerve stimulation induced release of noradrenaline from the rabbit heart after inhibition of prostaglandin synthesis *Acta physiol scand* 1971 83 163—168

Vane J R Inhibition of prostaglandin synthesis as a mechanism of action for aspirin like drugs *Nature New Biol* 1971 *213* 232—235

Acta physiol scand 1973 87 573—574
From the Department of Physiology Karolinska Institutet Stockholm Sweden

On the Release of Prostaglandin E_2 from the Rabbit Heart Following Infusion of Noradrenaline

By

MARIANNE JUNSTAD and ÅKE WENNMALM

Prostaglandins (PG s) of the E series are released from sympathetically innervated tissues following nerve stimulation or infusion of noradrenaline (NA) (Gilmore Vane and Wyllie 1968) On the basis of studies in the isolated rabbit heart where the endogenous synthesis of PG s was inhibited by 5 8 11 14 eicosatetraynoic acid (Wennmalm 1971) it seems clear that the release of NA is restricted by endogenous ly synthesized PG s

Since it has been suggested (*cf* Hedqvist 1970 Wennmalm 1971) that endogenous PG s might exert the inhibiting link in a negative feed back regulation of the release of the sympathetic transmitter it was of interest to investigate if infusion of NA causes an overflow of PG s also in the rabbit heart In addition an attempt was made to influence this overflow of PG s caused by NA infusion by using different adrenergic blocking agents

The isolated rabbit heart was perfused with Tyrode s solution using the Langen dorff technique NA was infused through a cannula close to the aorta at a rate of 5 μg/min in 10 periods divided by 15 min intervals Lipids in the perfusate from the heart during the NA infusion periods were extracted as described elsewhere (Wennmalm 1971) and their biological activity was tested on an isolated superfused rat stomach muscle strip Phentolamine (7×10^{7} M) propranolol (7×10 M) methysergide (6×10^{7} M) and diphenyl hydramine (7×10^{7} M) were added to the Tyrode s solution superfusing the assay organ Some samples of the biologically active lipids were subjected to thin layer chromatography

Infusion of NA caused an overflow of biologically active lipids from the heart corresponding to 14 ± 3 ng (mean $\pm$ S E n = 7) PGE /μg NA infused Addition of alpha or beta adrenergic receptor blocking drugs (phenoxybenzamine 3×10^{6} M or propranolol 5×10^{6} M) did not significantly change the outflow of PG like sub stances the activity corresponding to 18 ± 4 (n = 4) and 15 ± 2 (n = 3) ng PGE / μg NA infused The spontaneous overflow of biologically active lipids in the perfusate was low and most often no activity was found in the perfusate from the heart collected between the NA infusion periods

In some experiments the rabbits were pretreated with 6 OH dopamine following the scheme given by Thoenen and Tranzer (1968) When the hearts from th

rabbits were infused with NA, the outflow of biologically active lipids corresponded to 15±5 (n = 4) ng PGE /μg NA infused The concentration of NA in another series of hearts pretreated identically with 6 OH dopamine was low (0 1 μg/tissue) compared to controls (0 9 μg/g tissue)

Thin layer chromatography of lipid extracts from the perfusate during NA infusion from untreated hearts from hearts perfused with alpha or beta adrenergic blocking agents or from hearts pretreated with 6 OH dopamine revealed that the biologically active lipids chromatographed like PGE No activity corresponding to PGE_1 was found

The results obtained show that infusion of NA in the rabbit heart causes an overflow of PGE in the perafusate thus confirming in this organ the results by Gilmore *et al* (1968) The source of the PGE_ is probably not the adrenergic nerve endings since an almost complete destruction of the nerves did not significantly alter the outflow of PGE

Administration of the beta adrenergic blocking agent propranolol in a dose sufficient to inhibit completely the mechanical response to infusion of NA caused no change in the overflow of PGE from the organ Thus the mechanical response of the effector cell is not a prerequisite for overflow of PGE The alpha adrenergic blocking agent phenoxybenzamine was used in this series since it has been shown earlier that this drug causes an increased release of NA (Wennmalm 1971) which was interpreted as a result of inhibition of the endogenous prostaglandin feed back This explanation is probably not valid since the overflow of PGE from the NA infused rabbit heart was unchanged in the presence of phenoxybenzamine

In conclusion infusion of NA in the isolated rabbit heart causes an overflow of PGE into the perfusate The prostaglandin does not derive from the adrenergic nerves and the release of the compound is mediated via receptors which are not influenced by alpha or beta adrenergic receptor blocking agents

This work was supported bv the Swedish Medical Research Council project 14X 3027 and by Magnus Bergvall s Stiftelse

References

GILMORE N J R VANE and J H WYLLIE Prostaglandins released by the spleen *Nature* (Lond) 1968 *218* 1135—1140

HEDQVIST P Studies on the effect of prostaglandins E1 and E2 on the sympathetic neuromuscular transmission in some animals tissues *Acta physiol scand* 1970 *79* Suppl 345

THOENEN H and J P TRANZER Chemical sympathectomy by selective destruction of adrenergic nerve endings with 6-OH dopamine *Naunyn Schmiedebergs Arch exp Path Pharmak* 1968 *261* 271—288

WENNMALM A Studies on mechanisms controlling the secretion of neurotransmitters in the rabbit heart *Acta physiol scand* 1971 *83* Suppl 365

Acta physiol scand 1973 87 575—576

From the Department of Pharmacology University of Helsinki Finland

Acetylcholinesterase Activity is Decreased after Sexual Maturation in Bovine Pineal Gland

By

SEPPO ARO HEIKKI KARPPANEN and ERIK KLINGE

Precocious puberty has been noted in about one third of all boys afflicted with pineal neoplasms (Kitay and Altschule 1954) In extensive studies the role of the pineal gland in the regulation of mammalian gonadal functions has been established (Reiter 1972) This gland uniquely produces melatonin (5 methoxy N acetyl-serotonin) (Wurtman Axelrod and Kelly 1968) In mammals melatonin exerts an inhibitory effect on the gonads by blocking the secretion of the luteinizing hormone from the pituitary Another pineal compound 5 methoxytryptophol specifically reduces the output of the follicle stimulating hormone (Fraschini Collu and Martini 1971) The activity of hydroxyindole O methyltransferase (HIOMT) and also the content of melatonin are considerably increased in pineals of rats kept in darkness (Wurtman *et al* 1968) Cholinergic mechanisms seem to be involved in the darkness induced increase of the HIOMT activity (Wartman *et al* 1969)

To get more information about the poorly known cholinergic components of the pineal gland cholinesterase (ChE) activities of the pineals of sexually mature Ayrshire bulls and cows (b wt 250—350 kg) and sexually immature calves of both sexes (b wt 30—80 kg) were determined Fresh glands were taken in June at 7 00—8 30 a m to avoid possible seasonal or circadian variations in ChE activities Using selective inhibitors and various choline esters as substrates the activities were determined essentially according to Augustinsson (1948) The details have been presented elsewhere (Klinge 1970)

Table I presents the ChE activities in the pineals of different age and sex groups In relation to the weight of tissue the rate of hydrolysis of acetyl β methylcholine (MeCh) that indicates the acetylcholinesterase (AChE) activity was clearly higher ($p < 0\,001$) in the pineals of calves of both sexes than in the pineals of sexually mature animals In adult bulls this activity was also significantly higher ($p < 0\,01$) than in adult cows The rate of hydrolysis of butyrylcholine (BuCh) indicating the activity of the nonspecific cholinesterase (nsChE) was somewhat slower ($p < 0\,05$) in the pineals of adult bulls than in the other groups The velocity of hydrolysis of ACh well correlated to that what could be anticipated from the individual activities of the AChE and the nsChE

TABLE I Rate of hydrolysis of some choline esters during incubation with homogenates of pineal glands The manometric technique was used and the amount of CO_2 evolved is expressed in µl/g of fresh tissue/h ± S E

	Weight of pineals (mg)	MeCh	BuCh	ACh	n
Female calves	130±17	730±100	1450±90	1240±140	7
Male calves	130±16	900±150	1380±220	1560±300	4
Cows	230±17	270±20	1430±100	580±70	11
Bulls	240±16	380±20	990±70	600±50	13

The nerve net is the only tissue in the pineal exhibiting AChE activity (Eranko, Rechardt and Cunningham 1970) Probably ACh also has the same location. The present results suggest that the cholinergic influence on the pineal cells is in sexually immature animals different from that in mature ones The cholinergic tone probably affects the formation of the gonadal inhibiting hormones i e of the 5 methoxyindoles (Wartman *et al* 1969) Our findings suggest that a change in pineal cholinergic tone might be one of the mechanisms by which this gland can participate in the regulation of sexual maturation

This study was supported by a research grant from the Yrjo Jahnsson Foundation Finland

References

AUGUSTINSSON K B Cholinesterases A study in comparative enzymology *Acta physiol scand* 1948 *15* Suppl 52

ERANKO O L RECHARDT and A CUNNINGHAM Light and electron microscopic histochemical observations on cholinesterase containing sympathetic nerve fibres in the pineal body of the rat *Histochem J* 1970 2 479—489

FRASCHINI F R COLLU and L MARTINI Mechanisms of inhibitory action of pineal principles on gonadotropin secretion In *The Pineal Gland* Ed. by G E W Wolstenholme and Julie Knight Churchill Livingstone Edinburgh and London 1971 259—273

KITAY J I and M D ALTSCHULE *The Pineal Gland—A Review of the Physiologic Literature* Harvard University Press Cambridge Mass 1954

KLINGE E The acetylcholine content and cholinesterase activity of the bull retractor penis muscle *Acta physiol scand* 1970 78 159—167

Balin and S Glasser Excerpta Medica, Amsterdam 1972 71—114

WARTMAN S A B J BRANCH R GEORGE and A NEWMAN TAYLOR, Evidence for a cholinergic

REITER R J The role of the pineal in reproduction In *Reproductive Biology* Ed by H

influence on pineal hydroxyindole O methyltransferase activity with changes in environmental lighting *Life Sci* 1969 8 1263—1270

WURTMAN R J J AXELROD and D E KELLY *The Pineal* Academic Press New York and London 1968